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Identification and Optimization of a Novel Inhibitor of Mitochondrial Calpain 10

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Abstract

Calpain 10 has been localized to the mitochondria and is a key mediator of Ca^{2+} induced mitochondrial dysfunction. A peptide screen followed by a series of modifications identified the homodisulfide form of CYGAK (CYGAK)$_2$ as an inhibitor of calpain 10, while showing no inhibitory activity against calpain 1. Methylation or truncation of the N-terminal cysteine significantly reduced the inhibitory activity of (CYGAK)$_2$ and inhibition was reversed by reducing agents, suggesting that CYGAK forms a disulfide with a cysteine near the active site. Data suggests CYGAK may be a P' calpain inhibitor and may achieve its specificity through this mechanism. CYGAK inhibited calpain activity in intact mitochondria, renal cells, and hepatocytes, prevented Ca^{2+} induced cleavage of NDUFV2, and blocked Ca^{2+} induced state III dysfunction. (CYGAK)$_2$ is the first P' specific calpain inhibitor and will be a valuable tool to prevent Ca^{2+} induced mitochondrial dysfunction and explore the function of calpain 10.

Keywords

Calpain 10; mitochondria; inhibitor; mitochondrial dysfunction; kidney

Introduction

Calpains are a family of cysteine proteases first described and purified by Dayton *et al* in 1976 [1,2]. The calpains are conserved among eukaryotes with various calpain genes expressed in fungi, *C. elegans, Drosophila*, and mammals [3]. Within the vertebrate family, there are 14 large subunit calpain isoforms and a 28 kDa small calpain subunit [3], some of which are ubiquitously expressed [4] and others display a more tissue-specific distribution [5-7]. The seemingly ubiquitous calpain family members consist of calpains 1, 2, 4, 5, 7, 9, and 10 [3, 8-12]. The calpain family also can be divided into what are termed *typical* and *atypical* calpains [3]. The typical calpains contain a penta-EF hand Ca^{2+} binding motif in domain IV and are comprised of calpains 1, 2, 3, 8, 9, 11, 12, 13, and 14. The atypical calpains lack a penta-EF hand domain and consist of calpains 5, 6, 7, 10, and 15 (SOLH) [13-15].

Calpains have a number of physiological roles, including cytoskeleton remodeling, cell migration, embryonic development, and platelet function [16-19]. Calpains also play

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pathological roles in acute organ failure (e.g. liver, kidney, spinal cord injury, and stroke) and Alzhemeimer's disease as a mediator of apoptotic and necrotic cell death [20].

Calpain 10 has come to the forefront of calpain research because it has been identified as a diabetes mellitus type-2 susceptibility gene [21-26]. It may play a role in insulin secretion and trafficking as part of its regulation of cytoskeletal changes [22, 27]. More recently, we identified calpain 10 as the predominant mitochondrial calpain [28]. Over-expression of calpain caused mitochondrial dysfunction while calpain 10 depletion caused epithelial cell death (28, unpublished observations). Furthermore, mitochondrial calpain 10 mediates Ca^{2+}-induced electron transport chain dysfunction through the cleavage of NDUFV2 and ND6 and inhibition of complex 1 activity [28, 29]. Development of effective and specific inhibitors of calpain isoforms may therefore be useful as therapeutics for a number of calpainopathies.

Calpain inhibitors found in nature were discovered and isolated from the *Streptomyces* species. Examples of this generation of inhibitors include leupeptin [30] and antipain [31]. However, these peptide aldehydes lack cell permeability and/or selectivity for calpains over other protease systems. Subsequent investigation of these peptidyl aldehydes resulted in improved inhibitor potency through structure-activity relationship analysis. Cell permeability and stability were enhanced by the addition of hydrophobic N-terminal capping groups [31]. The inhibitor derivatives generated by these efforts include calpeptin [32], MDL 28170 (**1**) [33], and calpain inhibitors I and II (Ac-Leu-Leu-Nle-H and Ac-Leu-Leu-Met-H) [34].

Most investigators have endeavored to design peptide mimetics containing chemical 'warheads' that replace the scissile amide bond with an electron deficient center that can either bind reversibly or irreversibly with the thiolate moiety of the active site cysteine [31]. Electrophilic carbonyl-containing functional groups that have been used as chemical 'warheads' include α-diketones [35], α-keto phosphorous compounds [36], aldehydes [32-34, 37-39], and α-keto carbonyl compounds such as α-ketoacids, α-ketoamides, and α-ketoesters [35, 40-42]. It was later discovered that irreversible inhibitors could be produced by replacing the scissile bond of calpain substrates with methylsulfonium salts [43-45], disulfide linkages [46], epoxysuccinates and their derivatives, vinyl sulfones [47], and ketomethylenes [45, 48-52]. Of note, a number of 'non-warhead' peptide biphenyl hybrid compounds have been synthesized, the most potent of which exhibits an IC_{50} of 87 pM [53, 54]

A good example of nonpeptide calpain inhibitors is the α-mercaptoacrylates [31] described by Wang *et al* in 1996 [55]. The most potent α-mercaptoacrylates, PD150606 (**2**) and PD151746 (**3**), were not only cell permeable, but were 600-fold more selective for calpain over cathepsin B [55]. This selectivity is thought to be derived from the novel binding site of the α-mercaptoacrylates as compared to active-site calpain inhibitors.

Ultimately, all of the calpain inhibitors identified to date suffer from lack of potency, specificity between calpains and other cysteine proteases, specificity among calpain family members and/ or lack of cell permeability. The lack of selectivity among calpain isoforms has made it difficult to determine which specific isoforms, such as mitochondrial calpain 10, are responsible for various physiological and pathological responses. Thus, we have endeavored to develop an inhibitor that is both potent and selective for calpain 10. Peptide screening identified a mitochondrial calpain 10 inhibitor, which was then characterized and refined and examined for its ability to inhibit Ca^{2+}-induced mitochondrial dysfunction in renal cortical mitochondria.

Materials and Methods

Materials

Unless stated otherwise, all chemical reagents were obtained from Sigma-Aldrich, St. Louis, MO. Female New Zealand white rabbits (1.5-2.0 kg) were purchased from Myrtle's Rabbitry, Thompson Station, TN. Percoll® was obtained from Amersham Biosciences, Piscataway, NJ. The calpain substrate, Suc-Leu-Leu-Val-Tyr-AMC (sLLVY-AMC), was purchased from Bachem, King of Prussia, PA. Calpeptin and purified calpain 2 from porcine kidney were obtained from EMD Biosciences, La Jolla, CA. All peptides were purchased as custom syntheses from Anaspec, San Jose, CA or synthesized in our laboratory

Mitochondrial Isolation, Purification and Subfractionation

Kidney mitochondria were isolated as previously described[28] and suspended in 130 mM KCl, 9 mM Tris-PO$_4$, 4 mM Tris-HCl, 1 mM EGTA, (pH 7.4) with the addition of 5 mM pyruvate and 5 mM malate (Buffer A). Crude rabbit renal cortical mitochondria were layered onto a Percoll®/sucrose gradient and centrifuged at 20,000 g for 20 minutes. Following purification, mitochondria were sub-fractionated as described previously[28]. Purified matrix fraction was stored at −80°C until use.

Matrix Calpain 10 and Calpain 1 Activities

Calpain activity was determined by spectrofluorometrically using the calpain substrate sLLVY-AMC (50μM.) as previously described[28]. Mitochondrial matrix or respiring mitochondria were incubated with various concentrations of inhibitors and calpain 10 activity was measured under linear conditions as a function of AMC hydrolysis. Calpain 1 activity was determined as described above using purified calpain 1. In experiments reflecting calpain activity in the presence or absence of Ca^{2+}, either EGTA (1mM) or Ca^{2+} (1μM) was added to activity buffer prior to the addition of mitochondrial matrix. Matrix was then added to buffer followed by the addition of inhibitor and substrate.

Measurement of Cellular Calpain

Calpain activity was measured spectrofluorometrically using the calpain substrate, SLLVY-AMC, as previously described [28, 56]. Cellular suspensions (both NRK-52E cells and hepatocytes) were diluted in an isotonic calpain activity buffer (150 mM KCL, 20 mM HEPES, pH 7.4) and assayed for sLLVY-AMC (100 μM) cleavage in a 96-well plate (5×10^4 cells/well) for 20 minutes. In all cases, activity was measured under linear conditions as a function of sLLVY-AMC hydrolysis using excitation and emission wavelengths of 355 nm and 444 nm respectively.

Mitochondrial Respiration

Oxygen consumption was monitored using a YSI Model 5300 Biological Oxygen Monitor (YSI Incorporated, Yellow Springs, OH). RCM were suspended at approximately 1.3 mg protein/ml in Buffer A. The respiration chamber was maintained at 37° C and stirred magnetically. After the basal rate (state 4) of oxygen consumption was measured, ADP was added to a final concentration of 1 mM to measure state 3 respiration. For mitochondrial protection experiments, respiration measurements were performed following 30 minutes of incubation with calpain inhibitors and a five min exposure to 1 μM Ca^{2+} or diluent.

Immunoblotting

Mitochondria were boiled for 10 minutes and then subjected to SDS/PAGE and transferred to nitrocellulose membranes. Membranes were incubated with a primary antibody to NDUFV2 (A generous gift from Dr. Yamaguchi, The Scripps Research Institute, La Jolla, CA). Antibody

incubation was followed by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz, 1:1000 dilution). Immunoreactive protein was visualized by enhanced chemiluminescence (Pierce) and imaged using an Alpha Innotech imaging station.

Peptide Synthesis

Peptides were synthesized manually using FMOC amino acids and HCTU coupling. Rink amide MBHA resins (0.1 mmol, 139 mg) were swollen in NMP for two hours and then treated with 20% (v/v) piperidine in NMP (2 min × 5) and washed with NMP (1 min, 5 times). Amino acids were coupled following activation with HCTU (0.5 mmol, 206 mg) and DIEA (0.2 mL). Kaiser tests were performed to verify the completion of coupling to resins and subsequent amino acids respectively. FMOC protecting groups were removed with 20% (v/v) piperidine followed by washing with NMP. The peptides were cleaved with a TFA cocktail (TFA (80%, v/v), H_2O (5%, v/v), phenol (5%, w/v), triisopropyl silane (5%, v/v), anisole (5%, v/v)), for 2 h and were then precipitated into cold methyl tert-butyl ether. The suspension was kept at −81 °C for 30 min and centrifuged (~ 1000 g, 10 min). After decantation, the residue was dried and purified by reversed phase HPLC using a Waters C18 radial compression column with a H_2O/CH_3CN in 0.1% TFA gradient. The MW of the peptides were confirmed by MALDI mass spectrometry.

Renal Cells and Rat Hepatocytes

Normal rat kidney (NRK-52E) epithelial cells were cultured as previously described [57] in DMEM (high glucose) supplemented with 10% fetal calf serum. Cells were dislodged by gently scraping and pipetting to produce a homogenous suspension of cells. Following preparation of cell suspensions, cell viability was determined via trypan blue exclusion and only preparations with greater than 90% viability were used for analysis. Suspensions of rat hepatocytes were prepared from the liver of male Sprague-Dawley rats (200-250 g) by collagenase perfusion as previously described [58]. Only hepatocyte preparations yielding greater than 90% viability, as measured by the trypan blue exclusion assay, were used for experiments. The cells were suspended in Waymouth's MB-752/1 medium containing 10% fetal calf serum, 100 nM insulin, and 100 nM dexamethasone.

Statistics

NRK-52E cells from one passage, hepatocytes from one rat and RCM isolated from one rabbit represent one experiment (n=1). All data are expressed as means ± SE.

Results

Because Calpain 10 is a mitochondrial calpain and is responsible for mitochondrial calpain-like activity in RCM [28], mitochondrial matrix was utilized as the source of calpain 10.

An initial peptide screen identified the 6-mer CYGRKK as a potential calpain 10 inhibitor. To determine the amino acids that are critical for the calpain 10 inhibitory activity of CYGRKK, several modification schemes were utilized. To evaluate the role of the positively charged arginine, it was replaced with the inert amino acid alanine and incubated with mitochondrial matrix in the presence of the calpain substrate SLLVY-AMC. The potency of CYGAKK was greater than that of CYGRKK (IC_{50} of 90 nM versus 290 nM) (Fig 2a, table 1). Truncation of the C-terminal lysine, (i.e. CYGAK), had no significant effect when compared to CYGAKK (Fig. 1a, table 1); however, further truncation of the lysine (i.e. CYGA) resulted in complete loss of inhibitory activity (Fig. 1b, table 1). To determine if the positive charge on the lysine of CYGAK is required for inhibitory activity, the lysine was acetylated (CYGAK*). Surprisingly, the observed efficacy and potency of CYGAK* was no different than that of CYGAK, suggesting that the structural characteristics of the lysine side chain are necessary

and not the positive charge. To evaluate the role of the tyrosine in CYGAK, tyrosine was substituted with either valine (CVGAK) or alanine (CAGAK). Substitution with either valine or alanine significantly reduced the inhibitory activity of CYGAK, suggesting that tyrosine is critical for calpain inhibition (Fig. 1d, table 1).

We hypothesized that the N-terminal cysteine of the CYGAK was forming a sulfhydryl bond with the cysteine in or near the active site of mitochondrial calpain 10. The cysteine was removed (YGAK) or methylated (C'YGAK) and compared to CYGAK. Truncation of CYGAK to YGAK or methylation of the CYGAK cysteine (C'YGAK) completely abolished inhibitory activity, suggesting that the free sulfhydryl of cysteine in CYGAK is necessary for calpain inhibition (Fig. 1c, table 1).

Because the N-terminal cysteine is necessary for the inhibitory activity of CYGAK (Fig.1c), we determined if the activity of CYGAK was due to the formation of a mixed disulfide with cyteine in or near the active site of calpain 10. To accomplish this, mitochondrial matrix was pre-incubated for 15 min with CYGAK (1 μM) and calpain activity was then measured for 5 min. As expected, CYGAK pretreatment inhibited greater than 90% of calpain activity. The reducing agent β-mercaptoethanol (BME) (1 mM) or ditiothreitol (DTT) (1 mM) was added to matrix containing CYGAK for 5 min and calpain activity was again monitored for an additional 5 min. BME or DTT completely reversed the inhibitory activity of CYGAK (Fig. 2).

It was observed that CYGAK in aqueous solution readily forms a disulfide homodimer $(CYGAK)_2$. To determine whether mixed disulfides could be effective calpain 10 inhibitors, disulfides of CYGAK and methoxythiophenol (MeOPhs-sCYGAK) or ethanethiol (Ets-sCYGAK) were prepared. The MeOPhs-sCYGAK mixed disulfide was more potent than $CYGAK_2$ (IC_{50} of 56 nM versus 96 nM) whereas Ets-sCYGAK was significantly less potent (IC_{50} of 2000 nM) (Fig. 1e, table 1), which reflects the differences in the thiol pKa values (ethanethiol = 10.61, methoxythiophenol = 6.8).

Because most pharmacological inhibitors of calpain have historically shown minimal selectivity among calpain isoforms [41, 42], $CYGAK_2$ was tested against mitochondrial calpain 10 and purified calpain I. Although $CYGAK_2$ inhibited calpain 10, it had no inhibitory effect on calpain 1 at concentrations as high as 100 μM (Fig.3), identifying $CYGAK_2$ as a selective inhibitor of calpain 10.

Because Ca^{2+} increases the activity of most calpains, the inhibitory activity of $CYGAK_2$ was evaluated in the presence and absence of 6 mM Ca^{2+}. No changes in the inhibitory potency or efficacy of $CYGAK_2$ were observed in the presence or absence of Ca^{2+} (Fig. 4), suggesting that the binding and activity of $CYGAK_2$ to calpain 10 is Ca^{2+} independent.

To determine if $CYGAK_2$ is effective in whole mitochondria, isolated mitochondria were incubated with $CYGAK_2$ and calpain 10 activity was measured over time. $CYGAK_2$ was effective at inhibiting calpain 10 activity in whole mitochondrial with an IC_{50} of 200 nM (Fig. 5a), demonstrating that $CYGAK_2$ crossed mitochondrial membranes with an approximate two-fold loss of potency (100 vs. 200 nM).

We have shown previously that mitochondrial matrix calpain 10 was responsible for Ca^{2+}-induced cleavage of complex 1 proteins ND6 and NDUFV2, and inhibition of state 3 respiration [28]. Because $CYGAK_2$ was effective at inhibiting calpain activity in whole mitochondria, we sought to determine if $CYGAK_2$ was capable of blocking Ca^{2+} induced mitochondrial dysfunction. Isolated mitochondria were preincubated with calpeptin (10 μM) or $CYGAK_2$ (10 μM) and then treated with Ca^{2+} (1 μM) for 5 min. Pretreatment with both calpeptin and

CYGAK$_2$ blocked the Ca^{2+}-induced cleavage of the complex I subunit NDUFV2 (Fig. 5b), and it also blocked Ca^{2+} induced attenuation of state 3 respiration (Fig. 5c).

To evaluate the effectiveness of the calpain 10 specific inhibitor CYGAK$_2$ in cellular systems, SLLVY-AMC cleavage was monitored in suspensions of NRK-52E cells and hepatocytes. The calpain 10 inhibitor, CYGAK$_2$, inhibited cleavage of the substrate at 100 μM and 300 μM in both NRK-52E cells (10 and 18%) and hepatocytes (14 and 22%) (figure 6).

Discussion

To elucidate the pharmacophore in the initial inhibitor of mitochondrial calpain 10, CYGRKK, a series of substitutions were performed. Substitution of an alanine for arginine of CYGRKK significantly improved the potency of the inhibitor, demonstrating the arginine was non-essential for recognition by calpain 10. Additional experiments revealed that 1) the C-terminal lysine of CYGAKK was not needed to inhibit calpain 10, 2) the C-terminal lysine of CYGAK was needed to inhibit calpain 10 but the ε-amino group was not, 3) that the loss of tyrosine markedly decreased the inhibitory potency of CYGAK, and 4) that the loss of cysteine from CYGAK resulted in no inhibitory activity. Finally, methylation of the sulfhydryl group on cysteine in CYGAK completely blocked inhibition of calpain 10 by CYGAK. Thus, CYGAK$_2$ is an optimized inhibitor of mitochondrial calpain 10 with an IC$_{50}$ of approximately 100 nM. To confirm the selectivity of CYGAK$_2$, CYGAK$_2$ did not inhibit the ubiquitously expressed calpain 1 at concentrations up to 100 μM, 1,000 times greater that the IC$_{50}$ for calpain 10. To confirm that CYGAK$_2$ could cross mitochondrial membranes, it was shown that CYGAK$_2$ inhibits calpain 10 activity in charged mitochondria with an IC$_{50}$ of approximately 200 nM. In summary, CYGAK$_2$ is a potent and selective inhibitor of mitochondrial calpain 10 and is capable of crossing mitochondrial membranes. Because most of the calpain family members have not been characterized biochemically, we can not confirm that CYGAK$_2$, only inhibits calpain 10.

As stated above, removal of the cysteine from CYGAK or methylation of the sulfhydryl group, completely blocked the calpain 10 inhibitory activity of CYGAK, suggesting that the sulfhydryl group in the cysteine of CYGAK was necessary to inhibit calpain 10. Calpain 10 activity was restored by the reducing agents dithiothhreitol and β-mercaptoethanol, suggesting that the calpain inhibitory activity of CYGAK is through the formation of a mixed disulfide with a cysteine in or near the active site. While this is the first report using the reactive sulfhydryl of cysteine as a warhead for a calpain inhibitor, it is not surprising that a peptide that effects the sulfhydryl group in or near the active site of calpain 10 would be an effective inhibitor as sulfydryl modifying agents have been shown in the past to reduce the activity of calpains [62-64]. Interestingly, the addition of a reducing agent to calpain 10, does not significantly effect the activity of the enzyme. In contrast, the addition of similar concentrations of a reducing agent to calpain I resulted in an 11 fold increase in cleavage of sLLVY-AMC (data not shown), suggesting that the activity of these enzymes is differentially regulated by the oxidation state of the cysteines.

During our studies we determined that CYGAK readily forms a disulfide in solution. Thus, in all the above experiments, the calpain 10 inhibitors, except those lacking cysteine or a free-sulfhydryl group, were presumably disulfides. The thiol exchange reaction between a critical thiolate and peptide disulfide would be considerably faster than oxygen mediated oxidation of peptide thiol bound to the calpain. To test this mechanism, two mixed disulfides, MeOPhs-sCYGAK and Ets-SCYGAK, were synthesized. Both mixed disulfides showed significant inhibitory activity, which demonstrates that thiol exchange can occur. These observations also demonstrate that a single CYGAK monomer is sufficient for recognition. Indeed, the MeOPhs-sCYGAK peptide was nearly twice as potent as CYGAK dimer. It is notable that the MeOPhs-

sCYGAK peptide is about 100-fold more potent than the Ets-sCYGAK peptide. Based on the differences in pKa, the p-methoxythiophenol should be a susbstantially better leaving group than ethanethiol and CYGAK. We conclude that the mechanism for inhibition involves attack of a calpain thiolate on the cysteine sulfur and subsequent loss of the second thiol. The observation that the mixed disulfides did not inhibit calpain 1 suggests that only when bound to calpain 10 does the reactive thiolate have the appropriate distance and geometry to affect a nucleophilic substitution on a critical disulfide, which could be highly beneficial for specificity.

The peptide sLLVY-AMC and other calpain inhibitors based on the xLLxx motif such as ALLN, ALLM, and calpeptin are all recognized by calpains on the P-side of the scissile bond as demonstrated by the C-terminal liberation of AMC from sLLVY-AMC [59]. A P-side inhibitor, calpeptin (Z-Leu-Nle-CHO), is capable of inhibiting typical calpains with low nanomolar IC_{50}s (~40 nM, calpain I, ~60 nM calpain 2) (data not shown). In our previous studies we demonstrated that calpeptin inhibited mitochondrial calpain 10 with an IC_{50} of 4 nM [28] when using sLLVY-AMC as a substrate. These results support the idea that the P-side of calpains 1, 2, and 10 have similar structural features. Tompa et al. reported that calpain substrates have preferred amino acids in the P2, P1, P1′ and P2′ positions. For example, lysine, tyrosine and arginine are preferred in P1 and leucine, threonine, and valine at P2. In contrast, serine, threonine, and alanine are preferred in P1' and proline is preferred in P2′ [60]. Currier et al., designed a calpain 1 substrate with an optimal cleavage motif, P3 – PLFAER – P3′ [61]. Based on these studies, we would predict that tyrosine of CYGAK would be recognized at P1 and that glycine, alanine and lysine would occupy P1′, P2′ and P3′, respectively. Because MeOPhs-sCYGAK also potently inhibits calpain 10 acitivty, it is likely that the thiol exchange reaction is occurring with the CYGAK moiety situated primarily on the P' side of the active site in the canonical peptide orientation that allows for N-terminal thiol exchange to occur. A P' inhibitor is novel in that most peptide inhibitors of proteases achieve their specificity by binding to the P sites. In the proposed alignment, the cysteine would align with the P2 site and would inhibit calpain 10 through the interaction with a cysteine in or near the active site of calpain 10. Targeting P' interactions may therefore be an attractive target for achieving isoform specificity among other calpain inhibitors.

Arrington et al., identified mitochondrial calpain 10 as an important mediator of Ca^{2+}-induced mitochondrial dysfunction [28]. Inhibition of mitochondrial calpain 10 with calpeptin blocked the Ca^{2+}-induced cleavage of complex I subunits and Ca^{2+}-induced state III dysfunction [28]. Similar to what is seen with calpeptin, pretreatment of mitochondria with $CYGAK_2$ also protected mitochondria from Ca^{2+}-induced state 3 dysfunction and prevented the Ca^{2+}-induced cleavage of the complex I NDUFV2 subunit. An inhibitor such as $CYGAK_2$ that blocks Ca^{2+}-induced mitochondrial dysfunction in the setting of ischemia-reperfusion, drug or toxicant injury may have therapeutic potential. Furthermore, an isoform specific inhibitor of calpain 10 will limit unwanted toxicities secondary to the inhibition of typical calpains.

Additionally, we have shown that the novel calpain 10 specific inhibitor is effective at inhibiting the cleavage of the calpain substrate (sLLVY-AMC) in two cellular models. However, it should be noted that the concentrations required are 100-fold higher than that needed for isolated mitochondria, suggesting that this inhibitor does not readily cross the plasma membrane. Future studies will be directed towards increasing the cell permeability of $CYGAK_2$.

Supplementary Material

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Non-Standard Abbreviations

|	
(RCM)	Renal Cortical Mitochondria
(MeOPhs-sCYGAK)	disulfide of CYGAK-methoxythiophenol
(Ets-sCYGAK)	disulfide of CYGAK-ethanethiol
(sLLVY-AMC)	Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin
(DTT)	Dithiothreitol
(BME)	β-Mercaptoethanol

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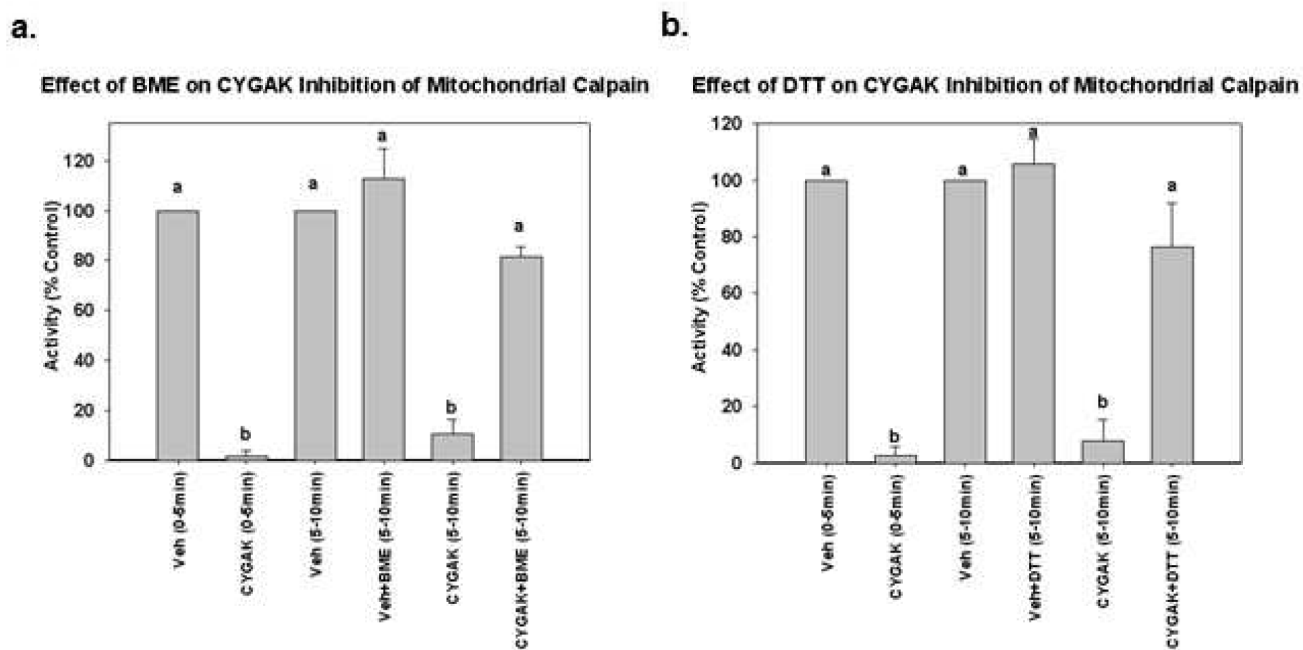
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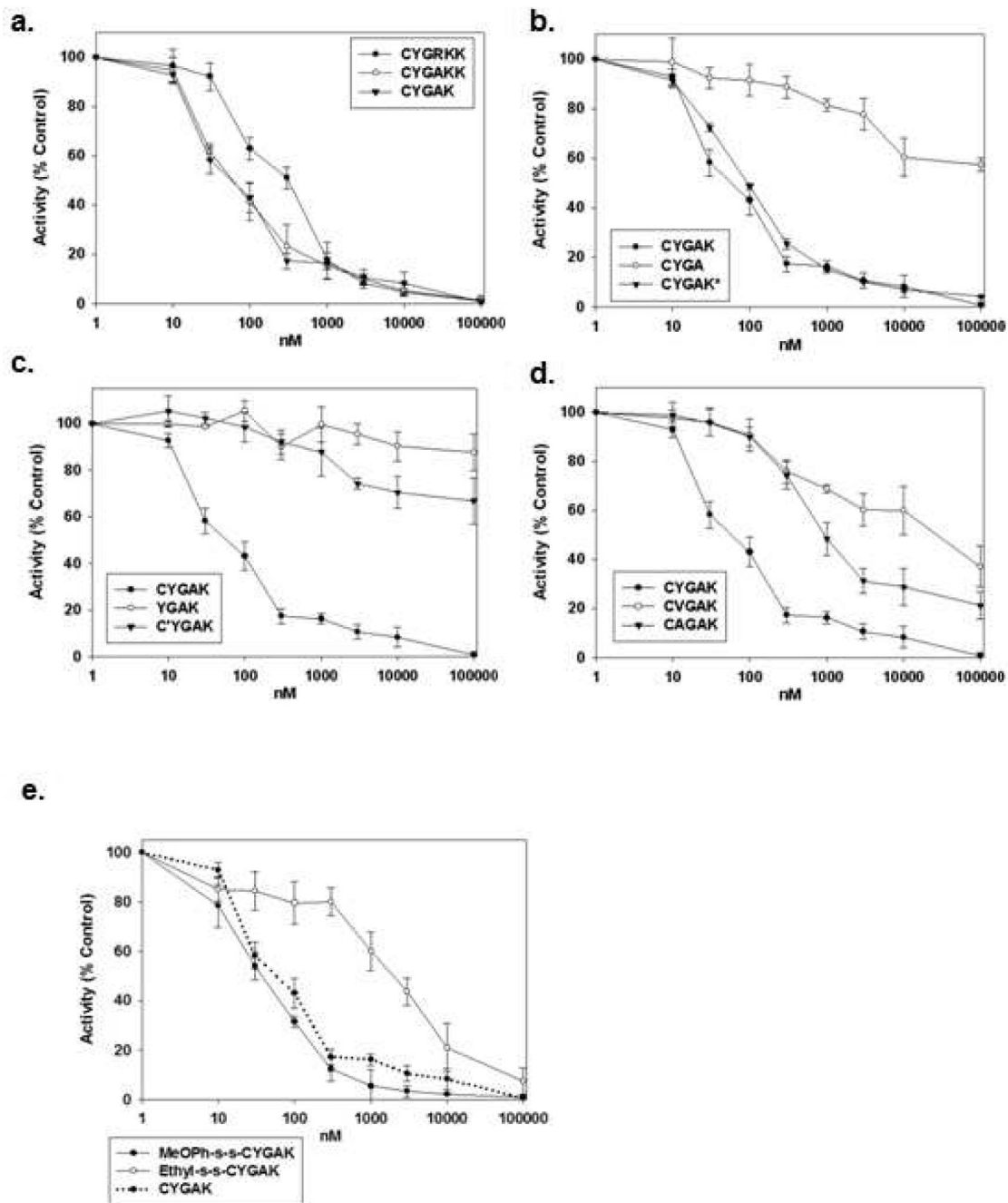
Figure 1. Optimization of the mitochondrial calpain 10 inhibitor
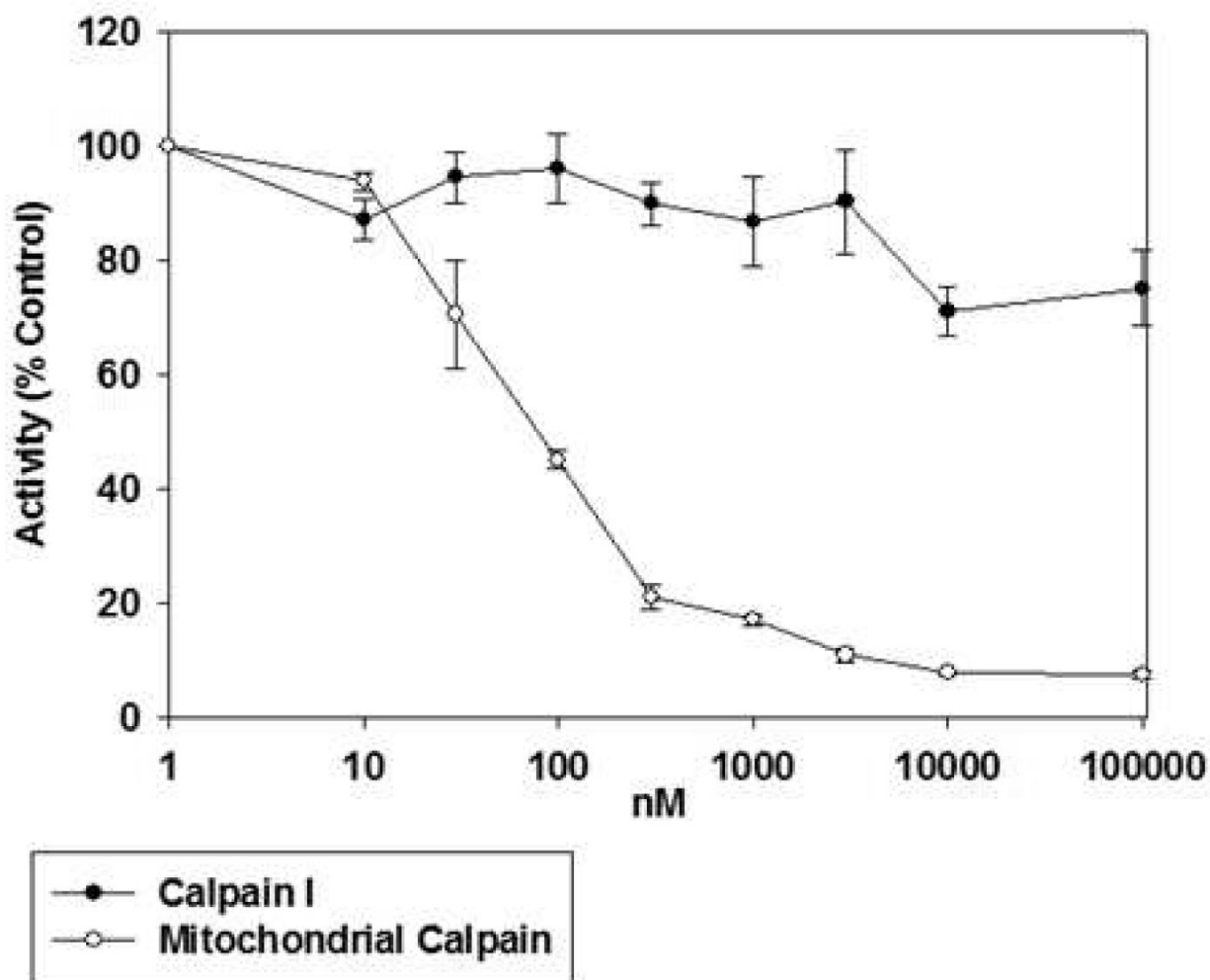The effect of increasing concentrations of peptides on mitochondrial matrix calpain 10 activity using sLLVY-AMC as a substrate. Data represent the means and SE (n ≥ 3).

a.

Effect of BME on CYGAK Inhibition of Mitochondrial Calpain

b.

Effect of DTT on CYGAK Inhibition of Mitochondrial Calpain

Figure 2. Reducing agents reverse CYGAK-induced inhibition of mitochondrial calpain 10 activity
Matrix calpain 10 activity was measured for 5 min in the presence and absence of CYGAK for 5 min. After 5 min the reducing agent BME (a) or DTT (b) was added and calpain activity was monitored for an additional 5 min. Data represent the means and SE ($n \geq 3$). Different subscripts are statistically different from each other; $p<0.05$.
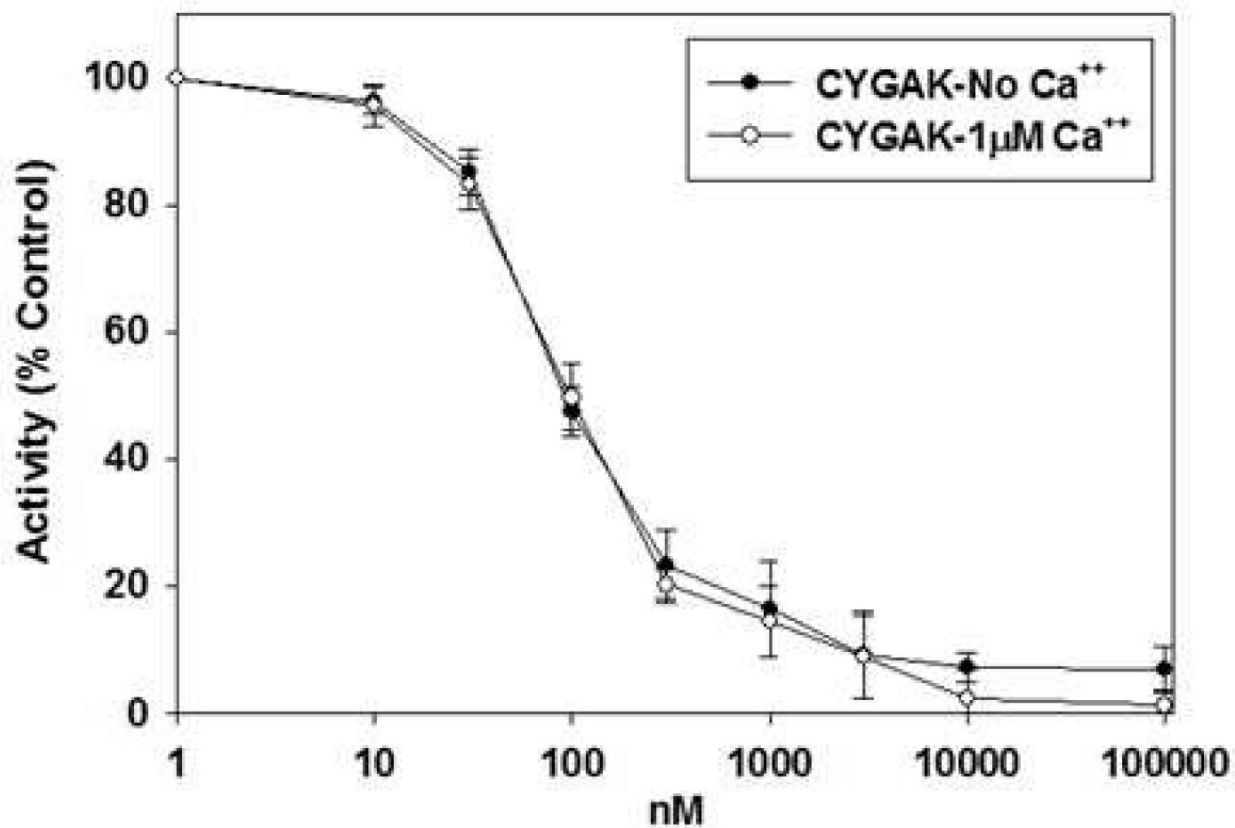
**Figure 3. CYGAK is selective for mitochondrial calpain 10**
Increasing concentrations of CYGAK were added to matrix calpain 10 or purified calpain 1
and sLLVY-AMC determined hydrolysis determined after 30 min. Data represent the means
and SE (n ≥ 3).

Figure 4. Inhibitory activity of CYGAK to calpain 10 is independent of Ca^{2+}
Inhibitory activity of CYGAK dimer was measured in the presence of EGTA or in the presence of $1\mu M$ Ca^{++}. Data represent the means and SE ($n \geq 3$).
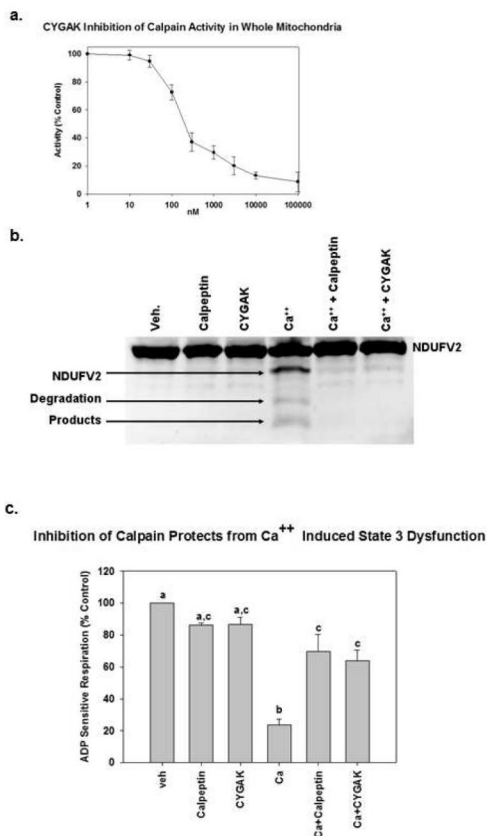
Figure 5. CYGAK inhibition of calpain activity in intact mitochondria prevents Ca^{++} induced cleavage of complex I proteins and state 3 dysfunction
Isolated RRC mitochondria were pretreated for 15 min with DMSO or the calpain inhibitors calpeptin (10μM) or CYGAK (10μM) and exposed to Ca^{2+} for 5 min. Mitochondrial damage was then assessed by measuring cleavage of the complex I subunit NDUFV2 by immunoblot (b) or by measuring state 3 respiration (c). Data represent the means and SE (N ≥ 3). Different subscripts are statistically different from each other; p<0.05.
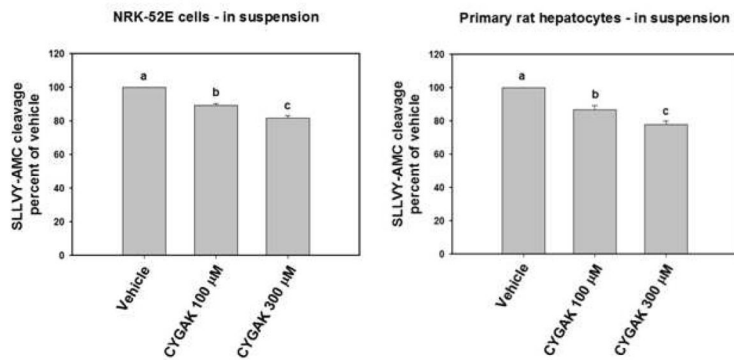
Figure 6. CYGAK inhibits calpain 10 activity in NRK52-E cells and isolated rat hepatocytes
NRK52-E cells and rat hepatocyte suspensions were incubated with SLLVY-AMC (100 μM) and either vehicle control or CYGAK at indicated concentrations for 20 min. Activity was measured under linear conditions and presented as percent of vehicle. Data represent the means and SE (n ≥ 3). Different lettered bars indicate statistically different means (p<0.05.

Table 1
Potency of inhibitors of mitochondrial calpain 10

The IC_{50} values of peptides that inhibit calpain 10 activity in mitochondrial matrix fractions using sLLVY-AMC. Data represent the means and SE's of at least 3 separate experiments. Different subscripts represent IC_{50}'s that are statistically different from each other; $p < 0.05$.

	Mean IC_{50} (nmol)
CYGRKK	290 ± 20 [a]
CYGRK	240 ± 70 [a]
CYGAK	100 ± 10 [b]
CYGA	> 100μM
YGAK	> 100μM
CYGAK	> 100μM
CVGAK	>40μM
CAGAK	2000 ± 140 [c]
MeOPh-s-s-CYGAK	60 ± 10 [d]
Ethyl-s-s-CYGAK	2000 ± 130 [c]
CYGAK*	90 ± 10 [b]
Calpeptin	4 ± 1 [e]