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Calpain 10 Homology Modeling with CYGAK and Increased Lipophilicity Leads to Greater Potency and Efficacy in Cells

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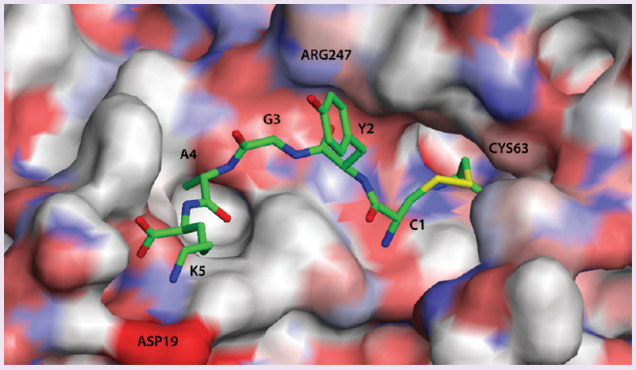
Supporting Information

ABSTRACT: Calpain 10 is a ubiquitously expressed mitochondrial and cytosolic Ca^{2+}-regulated cysteine protease in which overexpression or knockdown leads to mitochondrial dysfunction and cell death. We previously identified a potent and specific calpain 10 peptide inhibitor (CYGAK), but it was not efficacious in cells. Therefore, we created a homology model using the calpain 10 amino acid sequence and calpain 1 3-D structure and docked CYGAK in the active site. Using this model we modified the inhibitor to improve potency 2-fold (CYGAbuK). To increase cellular efficacy, we created CYGAK-*S*-phenyl-oleic acid heterodimers. Using renal mitochondrial matrix CYGAK, CYGAK-OC, and CYGAK-ON had IC_{50}'s of 70, 90, and 875 nM, respectively. Using isolated whole renal mitochondria CYGAK, CYGAK-OC, and CYGAK-ON had IC_{50}'s of 95, 196, and >10,000 nM, respectively. Using renal proximal tubular cells (RPTC) in primary culture, 30 min exposures to CYGAK-OC and CYGAbuK-OC decreased cellular calpain activity approximately 20% at 1 μM, and concentrations up to 100 μM had no additional effect. RPTC treated with 10 μM CYGAK-OC for 24 h induced accumulation of ATP synthase β and NDUFB8, two calpain 10 substrates. In summary, we used molecular modeling to improve the potency of CYGAK, while creating CYGAK-oleic acid heterodimers to improve efficacy in cells. Since calpain 10 has been implicated in type 2 diabetes and renal aging, the use of this inhibitor may contribute to elucidating the role of calpain 10 in these and other diseases.



Calpains are a family of Ca^{2+}-activated cysteine proteases that have been implicated in numerous cellular processes (cell signaling, apoptosis, and membrane rearrangement) and diseases (type 2 diabetes, Alzheimer's disease, myocardial infarcts, and acute kidney injury).[1−4] There are 15 mammalian calpains that are divided into two groups, typical and atypical. Typical calpains contain a penta-EF hand in domain IV, which is responsible for binding Ca^{2+}. Atypical calpains lack a penta-EF hand in domain IV.

Calpain 10 is a ubiquitously expressed atypical calpain found in the nucleus, mitochondria, and cytosol.[5−7] Our laboratory discovered calpain 10 in renal mitochondria of rabbits, rats, and mice.[7,8] In all of these species, only calpain 10 was detected in renal mitochondria. In contrast, other research groups have discovered calpain 1 and/or 2 in liver,[9−11] brain,[12] and heart[13] mitochondria. Mitochondrial calpain 10 activity primarily resides in the mitochondrial matrix and has been shown to decrease state 3 respiration after Ca^{2+} overload by cleaving NDUFB8, NDUFV2 (complex I proteins), and ATP synthase β.[7,14] Overexpression or knockdown of calpain 10 caused cell death,[7,15,16] demonstrating that cells require a specific amount of calpain 10 for viability.

While there are a large number of calpain inhibitors,[17] most bind to the active site cysteine to block activity, and thus many calpain inhibitors also inhibit other cysteine proteases.[18,19] Additionally, the calpain family shares a similar catalytic domain/active site limiting the selectivity of inhibitors for one calpain isoform over another. For example, peptide aldehyde inhibitors (calpeptin, *N*-acetyl-L-leucyl-L-leucyl-L-methioninal (ALLM) and *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN)) inhibit calpains and cathepsins similarly and are not specific for one calpain isoform. Calpain inhibitors such as calpeptin, ALLM, and ALLN bind the P-side of calpains, suggesting that members of

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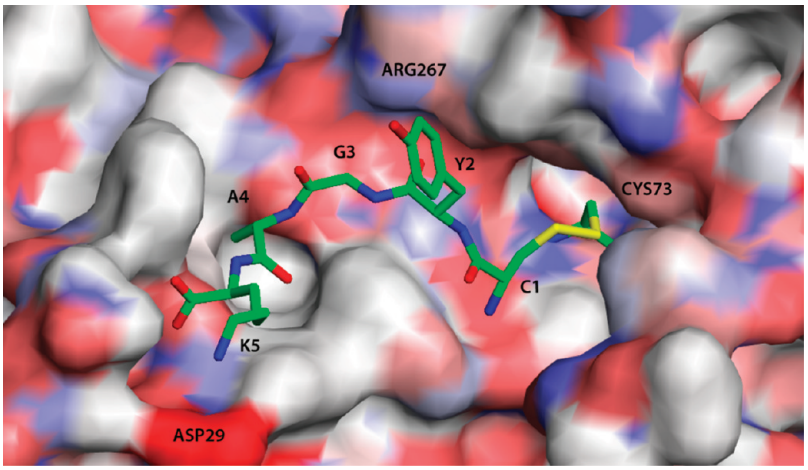

Figure 1. Creation of a homology model to improve CYGAK potency. The amino acid sequence for calpain 10 was used with the 3-D structure of calpain 1 (PDB: 1TL9), and CYGAK was docked in to the active site. The molecular surface of calpain 10 is colored by electrostatic potential, and the peptide residues and key active site residues are labeled by one and three letter codes, respectively.

the calpain family have a similar P-side structure.[20] PD150606 is selective for typical calpains because it binds to the penta-EF hand in domain IV, which is absent in atypical calpains.[21] These limitations demonstrate a need to develop calpain family and isoform specific inhibitors.

Our laboratory created a calpain 10 specific peptide inhibitor (CYGAK) by screening a peptide library and optimizing the lead hit.[22] We demonstrated that CYGAK had an IC_{50} of ~100 nM in mitochondrial matrix. Additionally, we showed that CYGAK had little effect on purified calpain 1 activity and was able to prevent mitochondrial calpain 10 substrate cleavage and a reduction in state 3 respiration after Ca^{2+} overload. We hypothesized that the selectivity of CYGAK was related to it being a P′-side inhibitor, unlike most calpain inhibitors. Two other examples of P′-side inhibitors are Ca-074 (an epoxide) and peptidyl α-ketoamides, which bind both the P and P′ sides of the calpain active site.[23,24] Unfortunately, the potency and efficacy of CYGAK in NRK-52E cells and rat hepatocytes was poor. Thus, the following research used a homology model to elucidate more information about the active site of calpain 10 and improve the potency of CYGAK. Also, to increase potency and efficacy in cells we created CYGAK-*S*-phenyl-oleic acid heterodimers.

RESULTS AND DISCUSSION

Molecular Modeling of CYGAK Leads to an Improved Calpain 10 Inhibitor. Even though calpain 1 and calpain 10 are in different calpain groups based on structure, there is a sufficient degree of sequence similarity in the catalytic domain (domain II) to allow template-based modeling. For example, the active site residues for calpain 10 are C73, H238, and N263, while the active site residues for calpain 1 are C115, H272, and N296.[2,3] Since the 3-D structure of calpain 10 is unknown, a homology model was generated using calpain 1 as a template (PDB: 1TL9).[25] After generation of the calpain 10 model structure, CYGAK was docked into the active site. In generating and validating the model, the structure−activity relationship previously established for the inhibitor series was considered.[22] In particular, a binding mode for CYGAK was generated in which the interactions of Y2 and K5 were consistent with loss of activity of CAGAK and CYGA peptides (Figure 1). From the model, it was observed that Y2 contributes van der Waals contacts to A229 and W265 and that K5 forms a saltbridge with D29. These nonbonded interactions are therefore in line with reported SAR for alanine substitution of the tyrosine and deletion of the C-terminal lysine. Furthermore, examination of the corresponding residues in the calpain 1 template crystal structure after sequence alignment indicates a structural basis for the selectivity of CYGAK for calpain 10. In particular, D29 is a lysine in calpain 1, suggesting an electrostatic repulsion with the critical peptide lysine that would preclude binding of the inhibitor. Additionally, the model revealed that CYGAK binds to the calpain 10 active site on the P′-side and not the P-side like most calpain inhibitors. This also may provide some selectivity for calpain 10 over calpain 1.

To further test and validate the homology structure for calpain 10, the activity of peptides incorporating specific substitutions was undertaken. Close examination of the peptide interacting residues suggested two modifications to the peptide sequence that would probe the model and allow further refinement of the side chain positions. In the first instance, it was proposed that substitution of A4 with α-aminobutyric acid (CYGAbuK, Figure 2)

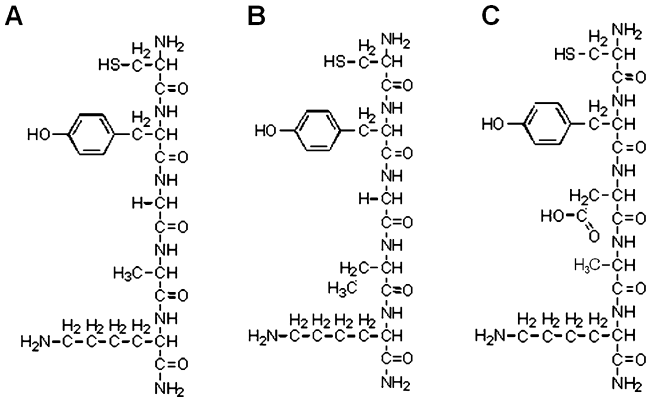

Figure 2. Structures of calpain 10 peptide inhibitors. The structure of the calpain 10 peptide inhibitor, CYGAK (A), along with two modified peptides developed from the homology model, CYGAbuK (B) and CYDAK (C).

would allow greater complementarity with a hydrophobic pocket close to the peptide C-terminus. In addition, proximity of R267 to G3 suggested that incorporation of an acidic side chain into the inhibitor (*i.e.*, CYDAK) may enable salt bridge formation.

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CYDAK and CYGAbuK were tested in mitochondrial matrix to determine if these inhibitors were more effective than CYGAK. As shown in Figure 3A, we determined that CYDAK

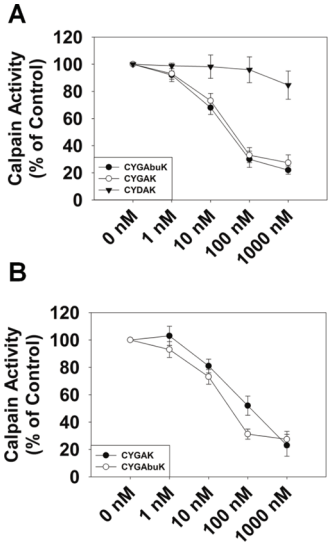

Figure 3. CYGAbuK is more potent than CYGAK in mitochondrial matrix and isolated mitochondria. (A) Mitochondrial matrix and (B) isolated mitochondria calpain activity assays were performed with 50 μM SLLVY-AMC in the presence of increasing concentrations of CYGAbuK, CYDAK, and CYGAK. The IC_{50}'s for each compound and a Student's *t* test were used to determine statistical significance with a sample size $\geq$3 and a *p*-value $\leq$0.05 required for significance.

was considerably less effective (175-fold) as a calpain 10 inhibitor ($CYDAK_{IC50}$ 14 $\pm$ 3 μM *vs* $CYGAK_{IC50}$ 80 $\pm$ 10 nM, whereas CYGAbuK was ~2-fold more potent than CYGAK ($CYGAbuK_{IC50}$ 44 $\pm$ 4 nM *vs* $CYGAK_{IC50}$ 80 $\pm$ 10 nM). We then tested CYGAbuK in whole mitochondria in the presence of metabolic substrates (malate/pyruvate). CYGAbuK was ~2-fold more potent in whole mitochondria than CYGAK (Figure 3B, $CYGAbuK_{IC50}$ 67 $\pm$ 8 nM *vs* $CYGAK_{IC50}$ 118 $\pm$ 12 nM). The rationale for the first substitution was therefore validated. The aspartic acid modification, however, was ineffective, suggesting that the conformational flexibility of the glycine residue allows adaptation into the binding site.

CYGAK-Oleic Acid Heterodimers and Inhibition of Calpain 10. Even though we were able to create a more potent calpain 10 inhibitor, CYGAbuK, potency and efficacy in cells remained a problem. While we did not study the mechanism of the ineffectiveness of CYGAK in cells, we previously showed that the disulfide bond is critical for calpain 10 inhibition.[22] Thus, we hypothesized that CYGAK was ineffective in cells because it was reduced in the cytosol, possibly by the glutathione system, and rendered inactive. It is also possible that CYGAK is too hydrophilic or degraded in the media.

Therefore, we created CYGAK-oleic acid heterodimers to increase lipophilicity (Figure 4) and act as a carrier molecule for CYGAK. Previously we showed that CYGAK monomers quickly form homodimers in solution, and all experiments are the result of homodimer exposure.[22] Additionally, we have shown that a heterodimer (*i.e.*, MeOPh-CYGAK) is a more potent calpain inhibitor than CYGAK, and thus use of a heterodimer should not greatly impact inhibition. Thus, we synthesized one CYGAK-oleic acid heterodimer that contained a cleavable ester bond between the oleic acid and the phenyl group and CYGAK (oleic acid cleavable, CYGAK-OC), while the other, CYGAK-ON (oleic acid noncleavable) contained a noncleavable ether bond.

In mitochondrial matrix, there were no differences in the potencies between CYGAK and CYGAK-OC ($CYGAK_{IC50}$ 70 $\pm$ 9 nM *vs* CYGAK-OC_{IC50} 90 $\pm$ 12 nM), but CYGAK-ON was less potent and effective than CYGAK (CYGAK-ON_{IC50} 875 $\pm$ 89 nM) (Figure 5A). Using whole mitochondria, there was no statistical difference between CYGAK and CYGAK-OC ($CYGAK_{IC50}$ 98 $\pm$ 23 nM *vs* CYGAK-OC_{IC50} 196 $\pm$ 30 nM) (Figure 5B). CYGAK-ON was ineffective in isolated mitochondria with an IC_{50} > 10,000 nM.

Because CYGAK is very potent and efficacious in isolated mitochondria, this small peptide with an overall +2 charge could be transported into the matrix through the TOM/TIM pathway.[26] Since many mitochondrial matrix proteins have presequences with an overall positive charge, it could explain why CYGAK is able to easily move to the mitochondrial matrix. CYGAK-ON was ~13-fold less potent than CYGAK in mitochondrial matrix and >100-fold less potent in isolated mitochondria, providing evidence that CYGAK-ON itself is effective in inhibiting calpain 10, albeit less potent, and has limited access to the mitochondrial matrix. In contrast, the equipotency of CYGAK and CYGAK-OC in mitochondrial matrix is likely the result of cleavage of the oleic acid prior to inhibition. We have previously shown that CYGAK does not inhibit calpain 1,[22] providing evidence that CYGAK is specific for calpain 10. Therefore, we determined the effects of CYGAK, CYGAK-OC, and CYGAK-ON in isolated cytosol, where calpains 1, 2, and 10 are available. It is important to note that SLLVY-AMC is not a specific calpain 10 substrate. In fact, SLLVY-AMC has been shown to be cleaved by many proteases including: the proteasome, calpains, Lon, ClpP, and cathepsins.[27−31] We detected no change in activity at any concentration for CYGAK, CYGAK-OC showed ~10% inhibition at 10 μM, and CYGAK-ON displayed ~10% inhibition at 1 μM and ~20% inhibition at 10 μM (Figure 5C). We used 10 μM calpeptin, a calpain and cysteine protease inhibitor, as a control in this experiment to determine how much activity is related to cysteine proteases. Calpeptin treatment blocked ~50% of the activity. These data provide evidence that CYGAK and CYGAK-OC do not significantly inhibit other cysteine proteases and that calpain 10 accounts for only a small portion of SLLVY cleavage in isolated cytosol.

Oleic Acid Heterodimers Inhibit Calpain 10 in Renal Proximal Tubular Cells. We hypothesized that an oleic acid heterodimer was a good delivery system to transport CYGAK into the cell due to fatty acid transport proteins on the cell and mitochondrial membranes.[32,33] Specifically, CD36 has been shown to be on the cell and outer mitochondrial membrane in the kidney.[34−37] Additionally, if the inhibitor is being reduced we thought that the addition of oleic acid may better protect the disulfide bond. Since CYGAK-OC was equipotent and efficacious as CYGAK in mitochondrial matrix and similar in isolated mitochondria, we tested these inhibitors in primary cultures of RPTC. We performed concentration−response experiments with CYGAK-OC and measured cellular calpain activity. Because CYGAbuK was a more potent inhibitor than CYGAK (Figure 3), we also tested this compound with a cleavable oleic acid. RPTC were treated with each compound for 30 min, and calpain activity was measured. CYGAK was only tested at 100 μM and did not inhibit calpain activity (Figure 6A). CYGAK-OC and CYGAbuK-OC equally inhibited calpain activity between 20% and

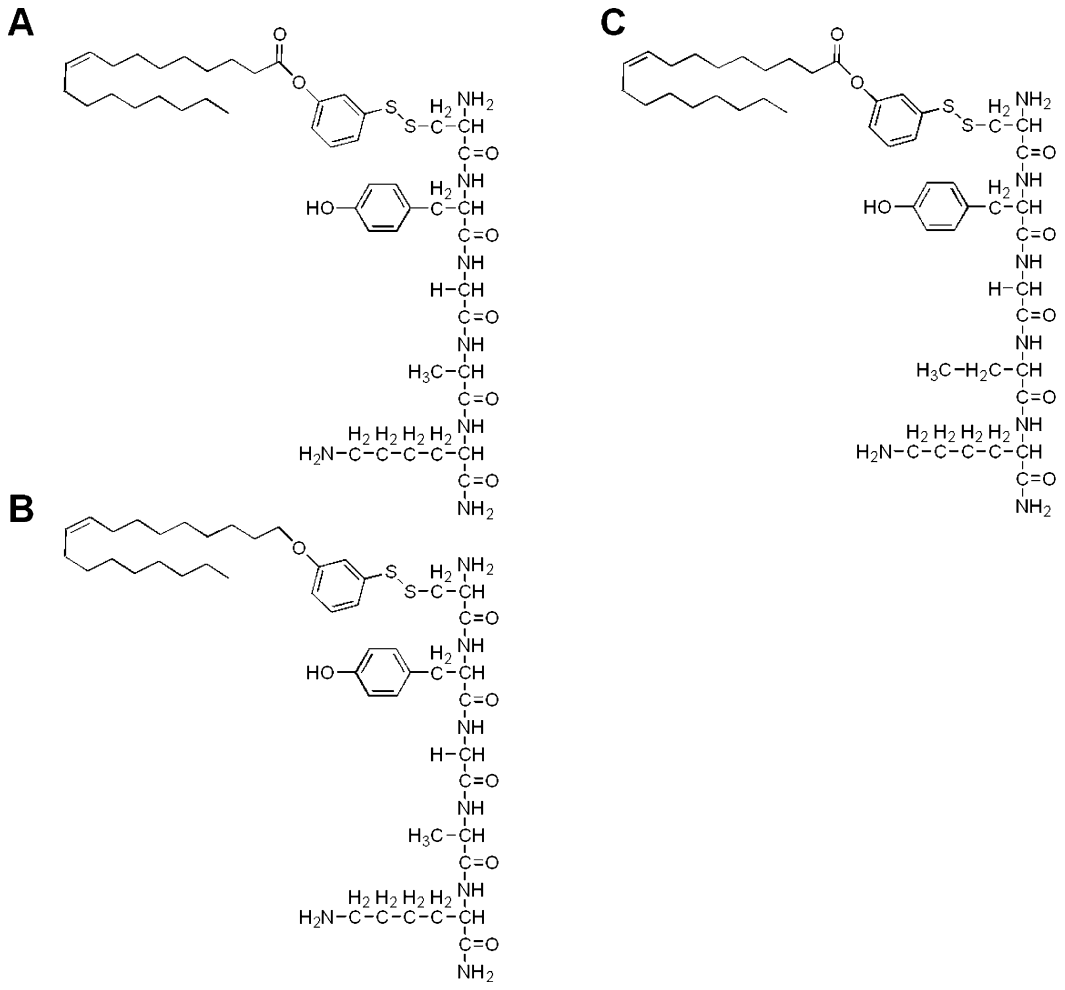

Figure 4. Structures of peptide-*S*-phenyl-oleic acid inhibitors. (A) CYGAK-OC (oleic acid cleavable), (B) CYGAK-ON (oleic acid noncleavable), and (C) CYGAbuK-OC.

30% at all concentrations tested. We performed the same experiment with a 60 min incubation period and detected 20−30% inhibition at 1−10 μM with both inhibitors (Figure 6B). At concentrations of 30 and 100 μM both inhibitors decreased calpain activity 30−40%. Finally, we determined if these inhibitors were effective after a 24 h incubation. In Figure 6C, we show that both inhibitors decreased calpain activity ∼40% at 100 μM. To ensure that the oleic acid portion of the inhibitor was not causing inhibition, we tested the OC homodimer at 100 μM at 30 min, 60 min, and 24 h and did not detect a decrease in RPTC calpain activity (Figure 6D).

CYGAK-OC and CYGAbuK-OC were equipotent inhibitors in RPTC even though CYGAbuK was 2-fold more potent than CYGAK when tested in mitochondrial matrix and isolated mitochondria. This is likely due to the fact that a 2-fold difference in potency in a more simplified system, mitochondrial matrix, may be lost in the complexity of cellular models.

As stated above, SLLVY-AMC is cleaved by many proteases including the proteasome, calpains, Lon, ClpP, and cathepsins.[27−31] This can increase the difficulty in interpreting calpain activity data. In addition, there is no active purified calpain 10 to test our compounds, so we used mitochondrial matrix or isolated mitochondria. The inability to purify calpain 10 that retains activity suggests that there is a critical interaction (protein and/or lipid) necessary for function. We have previously reported the limitations of using SLLVY as a measure of calpain activity.[38] When we first developed our calpain activity assays we tried several substrates, with good results being obtained with SLLVY.[7] Additionally, Rasbach *et al.* showed that in mitochondrial matrix and isolated mitochondria that CYGAK could inhibit ∼90% of all activity.[22] As expected with any exogenous substrate used in cells, we observed cleavage of SLLVY by other proteases.[38]

CYGAK-OC Inhibition of Mitochondrial Calpain 10. We determined if CYGAK-OC inhibited cytosolic or mitochondrial calpain 10 in RPTC. RPTC were treated with 1, 10, or 30 μM CYGAK-OC for 60 min followed by isolation of mitochondrial and cytosolic fractions. Activity was normalized to protein concentration. As shown in Figure 7A, 10 and 30 μM CYGAK-OC equally inhibited mitochondrial calpain activity by ∼40% but had no effect on cytosolic calpain activity. Mitochondrial and cytosolic fractionation purity was determined by immunoblotting for VDAC (mitochondrial) and GAPDH (cytosolic). Even though there was no decrease in cytosolic calpain 10 activity with CYGAK-OC treatment, it still may be inhibited, but the selectivity of SLLVY-AMC is not sufficient to detect inhibition. Since the mitochondrial fraction contains only three known SLLVY-AMC cleaving proteases (calpain 10, Lon, and ClpP),[7,29,31] calpain 10 contributes to more of the observed activity. These data confirm that mitochondrial calpain 10 activity is being inhibited at 10 μM CYGAK-OC in RPTC.

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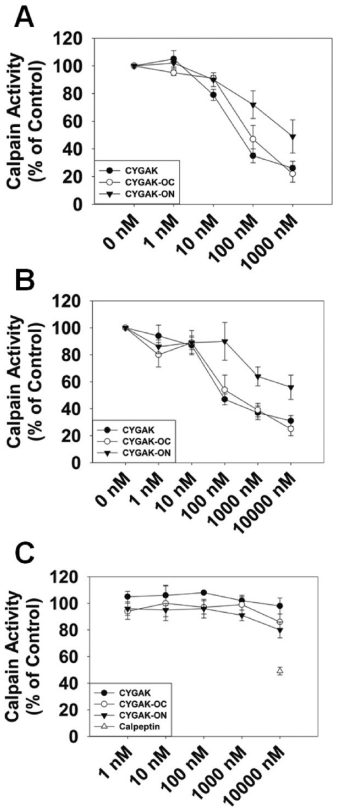


Figure 5. CYGAK-OC is equipotent to CYGAK in mitochondrial matrix and isolated mitochondria without any effect on cytosolic cysteine proteases. (A) Mitochondrial matrix, (B) isolated mitochondria, and (C) isolated cytosol calpain activity assays were performed with 50 μM SLLVY-AMC in the presence of increasing concentrations of CYGAK-OC and CYGAK-ON. The IC_{50}'s for each compound in A and B and the % inhibition in C were used in a Student's *t* test to determine statistical significance with a sample size $\geq$3 and a *p*-value $\leq$0.05 required for significance.

CYGAK-OC Inhibition of Mitochondrial Calpain 10 Induces Accumulation of Mitochondrial Calpain 10 Substrates. We have shown previously that calpain 10 degrades several proteins in the electron transport chain (NDUFB8, NDUFV2 (complex I), and ATP synthase β).[7] We have also shown that knockdown of calpain 10 results in the accumulation of these proteins.[15,16] Therefore, if CYGAK-OC is inhibiting mitochondrial calpain 10, then these proteins should accumulate. We treated RPTC for 24 h with 10 μM CYGAK-OC, isolated the mitochondrial fraction, and immunoblotted for ATP synthase β and NDUFB8. Indeed, we detected accumulation of ATP synthase β and NDUFB8 after CYGAK-OC treatment (Figure 8). Thus, 10 μM concentration of CYGAK-OC is sufficient to inhibit calpain 10 for 24 h in RPTC.

While only ~40% inhibition with CYGAK-OC treatment in RPTC was observed, this result is misleading because of the use of a promiscuous substrate, SLLVY (see above, Figure 6). It has been previously shown that SLLVY is cleaved by a number of proteases including cathepsins, proteasome, and the mitochondrial proteases Lon and ClpP.[27−31] We previously reported that ~50% of the cellular SLLVY cleavage is due to cysteine proteases in NRK-52E cells.[38] The other 50% of the cleavage has not been elucidated. Thus, one would expect that the maximal inhibition of SLLVY cleavage by CYGAK-OC would be less than calpeptin (*i.e.*, 50%). If CYGAK-OC inhibits ~40% of the total cellular SLLVY cleavage, then it is likely inhibiting most of the calpain 10 activity. The ~40% reduction in SLLVY hydrolysis in isolated mitochondrial fractions following CYGAK-OC exposure (Figure 7) is likely the result of ClpP and Lon protease cleavage of SLLVY. Most importantly, we detected an accumulation of mitochondrial calpain 10 substrates after a 24 h treatment with CYGAK-OC (Figure 8), demonstrating the selective inhibition of mitochondrial calpain 10.

In summary, this research demonstrates that molecular modeling can lead to improved calpain 10 inhibitors and reveals more information about the active site. CYGAK-S-phenyl oleic acid heterodimers were created and demonstrated to be effective in reducing mitochondrial calpain 10 activity in all calpain 10 model systems tested. Finally, treatment with CYGAK-OC produced the predicted biological effect in our RPTC by causing an accumulation of at least two mitochondrial calpain 10 substrates. Renal calpain 10 has been shown to be very important for renal cell viability,[15,16] and too much or too little calpain 10 results in cell death. Since much of the physiology and pathology of calpain 10 is unknown, a calpain 10 specific inhibitor that is effective in cells will allow the field to advance. Future use of this inhibitor will allow probing of additional physiological functions and greater understanding of the pathological role that calpain 10 plays in type 2 diabetes, aging, and acute organ injury.

METHODS

Reagents. The VDAC and ATP synthase β antibodies were purchased from Abcam (Cambridge, MA). The NDUFB8 antibody was obtained from Invitrogen (Carlsbad, CA). The GAPDH antibody was purchased from Fitzgerald (Acton, MA). The HRP-conjugated goat anti-rabbit/mouse secondary antibodies were obtained from Pierce (Rockford, IL). Calpeptin was purchased from Enzo Life Sciences (Plymouth Meeting, PA). SLLVY-AMC was obtained from Bachem (King of Prussia, PA). All other chemicals were purchased from Sigma (St. Louis, MO).

Molecular Modeling. A homology model for calpain 10 was generated using DiscoveryStudio 2.5 (Accelrys, San Diego, CA) through sequence alignment and model building and refinement based on the 1TL9 calpain 1 template structure. Modeled complexes of inhibitory peptides bound to calpain 10 were generated by manual docking of the CYAGK peptide into the P′ binding site of the protein after covalent bonding with the active site cysteine (C73). After atom typing and parametrization of the complex in the CHARMm forcefield, the Smart Minimizer algorithm composed of steepest descent and conjugate gradient and an implicit solvent model of Generalized Born with a simple Switching (GBSW) was applied. In general, all residues of the complex were flexible during calculations, and convergence to an energy minimum was achieved in less than 2000 steps. For generation of CYGAbuK and CYDAK peptide complexes, the desired residue was mutated and minimized using the protocol described above.

Peptide Synthesis. Standard solid phase peptide synthesis was performed to create CYGAK and CYGAbuK, as described previously.[22] Briefly, peptides were created with FMOC amino acids on a rink amide resin. Amino acids were activated by HCTU and coupled to the resin with DIEA. Kaiser tests were performed to ensure proper coupling. FMOC groups were removed by addition of 20% (v/v) of piperidine in NMP. Peptides were released from the resin with a TFA cocktail (80% TFA, 5% H_2O, 5% phenol, 5% triisopropyl silane, and 5% anisole v/v). Peptides were lyophilized and purified on a reverse phase HPLC.

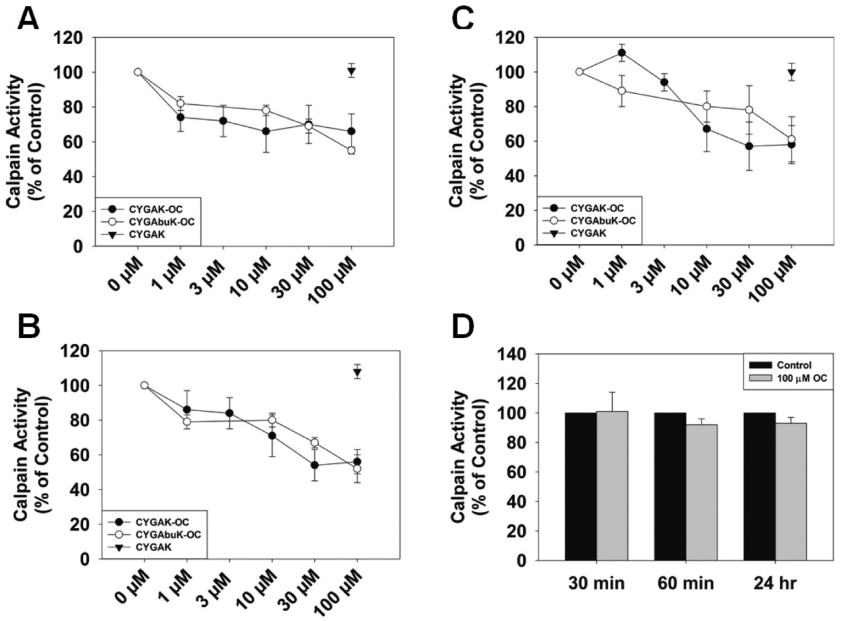
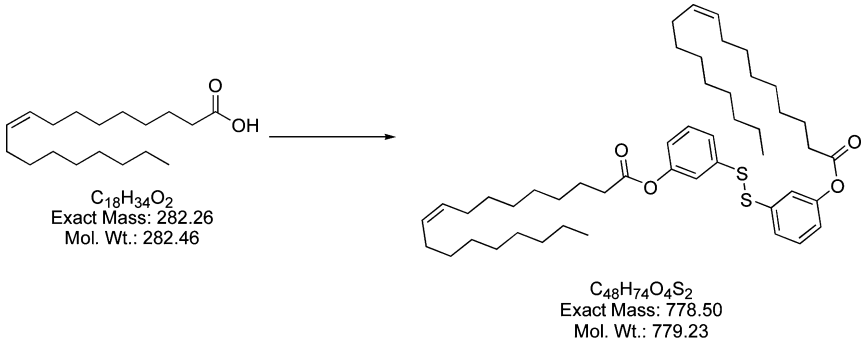
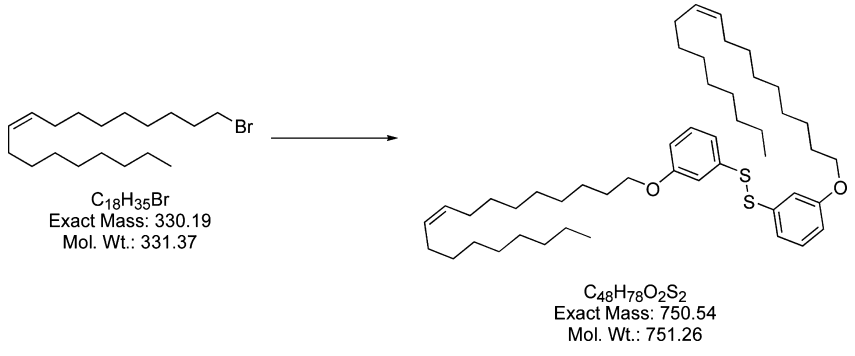


Figure 6. CYGAK-OC and CYGAbuK-OC inhibit calpain 10 activity in RPTC. (A) RPTC after a 30 min incubation, (B) RPTC after a 60 min incubation, and (C) RPTC after a 24 h incubation were assayed for calpain activity with 100 μM SLLVY-AMC in the presence of increasing concentrations of CYGAK-OC and CYGAbuK-OC. (D) RPTC after 30 min, 60 min, and 24 h incubation were assayed for calpain activity with 100 μM SLLVY-AMC with 100 μM OC or ON. Each concentration for each compound was compared to the control, and a Student's *t* test was used to determine statistical significance with a sample size $\geq$3 and a *p*-value $\leq$0.05 required for significance.



Oleic Acid Heterodimer Synthesis. To a flame-dried flask cooled under argon was added the oleic acid (1.0 g, 3.54 mmol, 0.2 M in CH_2Cl_2). While stirring at 0 °C oxallyl chloride (0.33 mL, 3.85 mmol) and 50 mL of DMF were added . After stirring for approximately 1 h, the solution was concentrated, 3,3′dihydroxydiphenyl disulfide was added (0.418 g, 1.67 mmol), and the mixture was taken up in 18 mL of CH_2Cl_2. While stirring at 0 °C NEt_3 (0.57 mL, 4.2 mmol) and 4-(dimethylamino)pyridine (0.024 g, 0.02 mmol) were added. The reaction was followed by TLC. Upon completion the reaction was diluted with water, and the two layers were separated. The aqueous layer was washed twice more with CH_2Cl_2, and the organic material was combined. The organic material was washed with brine, dried with Na_2SO_4, filtered, and concentrated. Purification *via* teledyne isco using silica gel afforded the desired material as a clear liquid using a gradient of 100% hexanes to 8:2 hexanes/EtOAc over 15 min.



To a flame-dried flask cooled under argon was added the oleic bromide (0.2 g, 0.603 mmol) and then phenol (0.0751 g, 0.3 mmol), K_2CO_3, (0.207 g, 1.5 mmol), NaI (0.045 g, 0.3 mmol), and 3 mL of dry DMF. The reaction was stirred at RT overnight in the dark. Upon completion (determined by TLC) the reaction was diluted with 10 mL of CH_2Cl_2, filtered, and concentrated. Purification *via* teledyne isco using silica gel afforded the desired material as a clear liquid using a gradient of 100% hexanes to 7:3 hexanes/EtOAc over 14 min.

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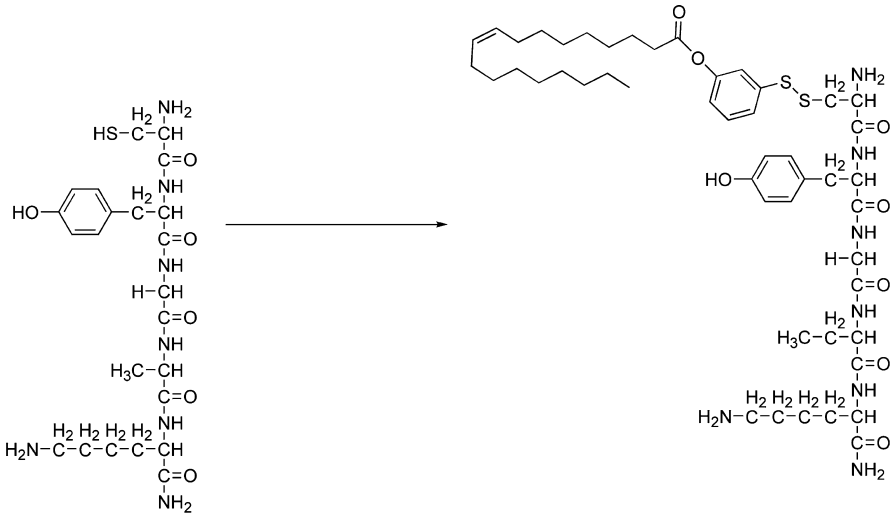

$C_{23}H_{37}N_7O_6S$
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Mol. Wt.: 539.65

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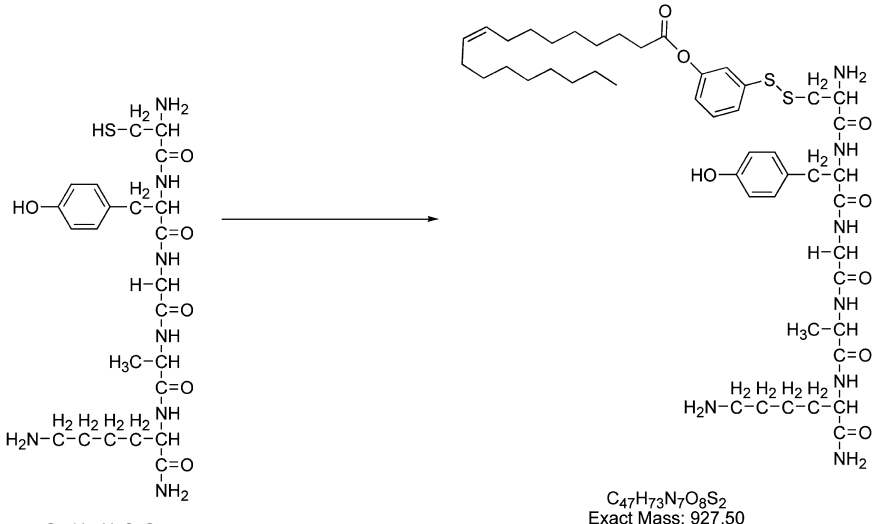
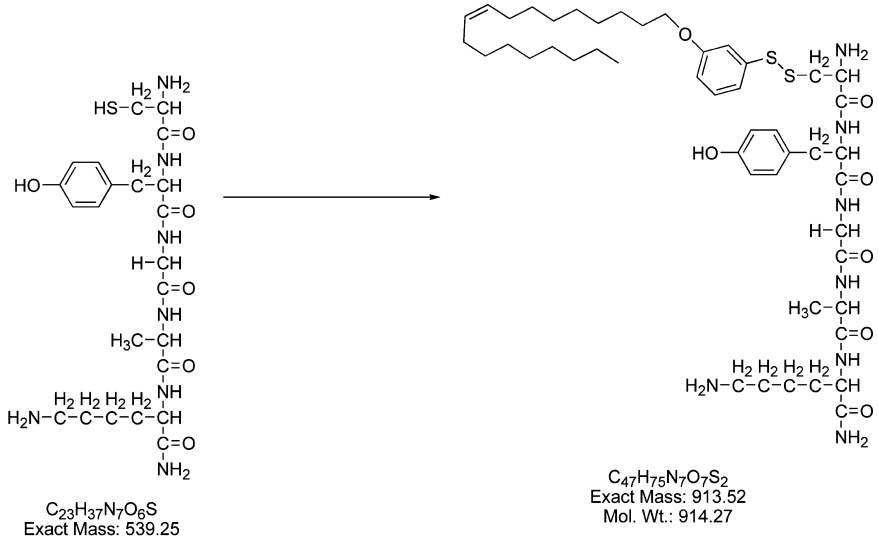
Mol. Wt.: 942.28



$C_{23}H_{37}N_7O_6S$
Exact Mass: 539.25
Mol. Wt.: 539.65

$C_{47}H_{73}N_7O_8S_2$
Exact Mass: 927.50
Mol. Wt.: 928.25



$C_{23}H_{37}N_7O_6S$
Exact Mass: 539.25
Mol. Wt.: 539.65

$C_{47}H_{75}N_7O_7S_2$
Exact Mass: 913.52
Mol. Wt.: 914.27

General Procedure for Heterodimer Formation. To a dry flask was added the homodimer (2.0 equiv, 0.1 M in $CHCl_3$). The crude material peptide was taken up as a 0.1 M solution in EtOH (dry) and added to the stirring chloroform solution. The homogeneous solution was stirred at RT under argon for 4 days. It was then purified by reverse phase HPLC (95:5 H_2O w/0.1% TFA/MeCN w/0.1% TFA to 100% MeCN w/0.1% TFA over 120 min) on a waters

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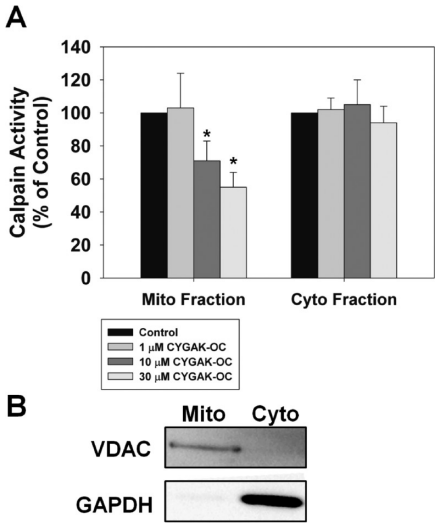

Figure 7. CYGAK-OC is inhibiting mitochondrial calpain 10. (A) RPTC were treated with 1, 10, or 30 μM CYGAK-OC for 60 min followed by isolation of the mitochondrial and cytosolic fractions, which were assayed for calpain activity. The activity values were normalized to protein concentrations. (B) Immunoblots for VDAC (mitochondrial) and GAPDH (cytosolic) show the purity of our isolation procedures. Each concentration for CYGAK-OC was compared to the control, and a Student's *t* test was used to determine statistical significance with a sample size ≥3 and a *p*-value ≤0.05 required for significance.

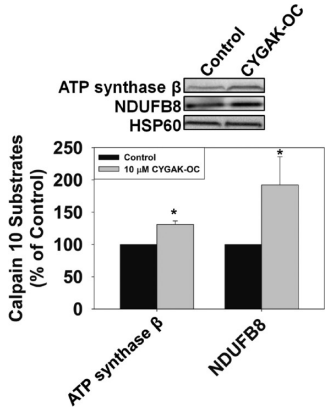

Figure 8. CYGAK-OC induces an accumulation of mitochondrial calpain 10 substrates. CYGAK-OC was treated on RPTC for 24 h at 10 μM followed by isolation of the mitochondrial fraction. Immunoblots were performed to for ATP synthase β, NDUFB8, and HSP60 (loading control). Densitometry was performed and graphed. A Student's *t* test was performed to determine statistical significance between CYGAK-OC and the control samples with a sample size ≥3 and a *p*-value ≤0.05 required for significance.

C18 column (flow rate, 6 mL/min). The desired material was frozen and lyopholyzied.

Isolation of Renal Cortical Mitochondria and Cytosol. Renal cortical mitochondria and cytosol were isolated from New Zealand White rabbits as described previously.[7,39] After the final centrifugation, the mitochondrial pellet was resuspended in mitochondrial isolation buffer + 5 mM malate and 6 mM pyruvate for calpain activity assays.

To isolate mitochondrial matrix, briefly, whole mitochondria were swollen in hypotonic swelling buffer (10 mM KH_2PO_4, pH 7.4) for 20 min and centrifuged at 10,000*g* for 15 min to pellet mitoplasts. Mitoplasts were resuspended in matrix buffer (300 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and sonicated to rupture the inner membrane. The mitoplasts solution was centrifuged at 100,000*g* for 30 min to pellet inner membrane with the matrix being the supernatant.[7,40]

RPTC. The iron oxide perfusion method was used to isolate renal proximal tubules from New Zealand White rabbits. Once isolated, the tubules were grown in glucose-free media for 6 days (until confluent) in either 96-well plates or 35 mm dishes, as described previously.[41,42]

Separation of Mitochondrial and Cytosolic Fractions from RPTC. After treatment with CYGAK-OC, RPTC were scraped in 75 μL of homogenization buffer (0.32 M sucrose, 50 mM Tris-HCl, 1 mM β-mercaptoethanol, 1 mM EDTA, pH 8.0). Samples were sonicated briefly followed by centrifugation at 900*g* for 10 min. The supernatant was transferred to another tube and centrifuged at 15,000*g* for 10 min. The supernatant (cytosol) was transferred to a new tube. The pellet (mitochondria) was resuspended in 20 μL of homogenization buffer.

Calpain Activity Assays. All assays were performed in black 96-well plates as previously described.[7,38] For rabbit mitochondria and cytosol, calpain activity buffer (150 mM KCl and 20 mM HEPES pH 7.4) was placed in the well, followed by mitochondria or cytosol, inhibitor, and 50 μM SLLVY. The spectrofluorometer measured increases in fluorescence every 30 s for 20 min. Confluent RPTC grown in 96-well plates were treated with CYGAK-OC, CYGAK-ON, or CYGAbuK-OC for 30 or 60 min or 24 h, and 100 μM SLLVY was added to start the assay. Confluent RPTC grown in 35 mm dishes were treated with 1, 10, or 30 μM CYGAK-OC. After 60 min, mitochondrial and cytosolic fractions were isolated, and calpain activity determined as described above. The purity of the fractionation was determined by immunoblotting for VDAC (mitochondrial marker) and GAPDH (cytosolic marker).

Immunoblot Analysis. RPTC mitochondrial and cytosolic fractions were electrophoresed on SDS-PAGE gels (4–12%) followed by transfer to nitrocellulose membranes. The membranes were blocked in 2.5% nonfat milk/TBST (Tris-buffered saline Tween 20) for 1 h. All primary antibodies were incubated on a shaker at 4 °C overnight. NDUFB8, ATP synthase β, and GAPDH were used at a 1:1000 dilution. VDAC was used at a 1:500 dilution. Secondary antibody (anti-mouse or anti-rabbit) was used at 1:10,000 dilution for 1 h at RT. An Alpha Innotech imaging system was used to visualize and quantify membranes for immunoreactive proteins using enhanced chemiluminescence detection.

Statistical Analysis. A one-way ANOVA with a Student–Newman–Keuls test was used to determine significance between multiple groups, and a Student's *t* test was used to determine significance between two groups. A single mitochondrial or cytosolic preparation from one rabbit kidney or RPTC prepared from one rabbit kidney is considered one experiment. The sample size for each group was ≥3, and a *p*-value ≤0.05 was required for statistical significance.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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