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Synthesis of Novel Sulfonamide-Based Calpain Inhibitors and Their Potential as Anti-Tumor Agents

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Abstract

Calpain is a class of intracellular cytoplasmic cysteine proteases.1 The enzyme participates in different intracellular signaling pathways that are mediated by Ca2+.2 The two major isoforms of calpain universally distributed in most mammalian tissues are calpain 1 (μ-calpain) and calpain 2 (m-calpain). The exact in vivo function of the enzyme is not clear, but calpain has been implicated in a variety of physiological and pathological conditions,3 such as cancer, stroke, cardiac ischaemia, muscular dystrophy, cataract and Alzheimer's disease. Calpain inhibitors are therefore of interest as therapeutic agents and as biomedical tools.

Several potent calpain inhibitors isolated from natural sources as well as synthesized in the laboratory have been reported (Chapter 1.4). Unfortunately, most of the inhibitors show poor calpain selectivity, metabolic stability and cell permeability. In an attempt to develop potential calpain inhibitors based on the X-ray crystal structure of the μ-calpain, NCI compound library was screened by virtual screening method and diazosulfonamide **1** (Ki = 1.0 ± 0.02 μM) was identified as a new nonpeptide competitive inhibitor of μ-calpain. Analogues of **1** were synthesized to explore structure requirements of **1** (Chapter 2). In order to test the hypothesis that derivatives of diazosulfonamide **1** with an electrophilic group can have a covalent interaction with cysteine at the active site of calpain, novel sulfonamide-based peptidomimetic analogues of **1** were synthesized and assayed for their ability to inhibit μ-calpain utilizing a kinetic fluorescence assay and for their anti-tumor ability by SRB colorimetric assay (Chapter 3). Introduction of the electrophilic functionality significantly enhanced calpain inhibition. From 13 target compounds, 7 compounds had better calpain 1 inhibition (Ki ranging from 9 to 500nM) than **1** and 5 showed good anticancer activity (GI50 ranging from 4 to 22μM). Sulfonamide-based peptidomimetic analogue **19** with Ki of 9 nM is over 100-fold more potent than the lead diazosulfonamide **1**. Compound **16** was the most effective anticancer agent (GI50 4μM) of the series.

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SYNTHESIS OF NOVEL SULFONAMIDE-BASED CALPAIN INHIBITORS AND THEIR POTENTIAL AS ANTI-TUMOR AGENTS

A Thesis
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By
Jin Xu
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DEDICATION

This thesis is dedicated to
all my family members and friends
who supported me with their encouragement and help.

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ABSTRACT

Calpain is a class of intracellular cytoplasmic cysteine proteases.[1] The enzyme participates in different intracellular signaling pathways that are mediated by Ca^{2+}.[2] The two major isoforms of calpain universally distributed in most mammalian tissues are calpain 1 (μ-calpain) and calpain 2 (m-calpain). The exact in vivo function of the enzyme is not clear, but calpain has been implicated in a variety of physiological and pathological conditions,[3] such as cancer, stroke, cardiac ischaemia, muscular dystrophy, cataract and Alzheimer's disease. Calpain inhibitors are therefore of interest as therapeutic agents and as biomedical tools.

Several potent calpain inhibitors isolated from natural sources as well as synthesized in the laboratory have been reported (Chapter 1.4). Unfortunately, most of the inhibitors show poor calpain selectivity, metabolic stability and cell permeability. In an attempt to discover new calpain inhibitors, structure-based virtual screening of the National cancer Institute's (NCI) led to the identification of diazosulfonamide **1** (Ki = 1.0 ± 0.02 μM) as a new nonpeptide competitive inhibitor of μ-calpain. Using **1** as our lead compound, we first explored the structural requirements of the compound that are important for potent calpain inhibition of calpain (Chapter 2). Secondly, derivatives of **1** were synthesized to test the hypothesis that introduction of an electrophilic group for covalent interaction with the catalytic site thiolate group of calpain would enhance inhibition of the enzyme. Thirdly, the anti-tumor potential of the compounds was determined by screening them against human and mouse melanoma cell lines (Chapter 3). Our data showed that the carboxylic acid and the thiazole groups of **1** are important for potent inhibition of calpain. It was also found that incorporation of electrophilic groups (aldehyde and alpha-ketoamide) into derivatives of **1** significantly enhanced μ-calpain inhibition with K_i values ranging from 9 nM to 500 nM). Sulfonamide-based peptidomimetic analogue **19** with K_i of 9 nM was the most potent calpain inhibitor of the series and was over 100-fold more potent than the lead diazosulfonamide **1**. Five of the compounds inhibited melanoma cell growth with GI_{50} values ranging from 4 μM to 22 μM. Compound **16** was the most effective anti-tumor agent (GI_{50} 4μM) of this series.

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LIST OF ABBREVIATIONS

Abu	2-Aminobutyric acid
AD	Alzheimer's disease
Ala (A)	Alanine
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4- propionic acid
Arg (R)	Arginine
Asn (N)	Asparagine
Boc	Butoxycarbonyl
BOP	Benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate
Br	Bromo
CaM	Calmodulin
cAMP	Cyclic 3',5'-adenosine monophosphate
Capn	Calpain gene
CATM	Cataract with microphthalmia
CDI	1,1-Carbonyldiimidazole
CDK (Cdk)	Cyclin dependent kinase
Cl	Chloro
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
Cys	Cysteine
DCC	N,N'-dicyclohexylcarbodiimide
DCM	Dichloromethane
DIPEA	N,N-diisopropylethylamine
DMEM	Dulbecco's Modification of Eagle's Medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
EC	Enzyme Commission
EDC	Ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ESI	Electron spray ionization
EST	Expressed Sequence Tags
EtOAc	Ethyl acetate
F	Fluoro
FBS	Fetal Bovine Serum
Gln (Q)	Glutamine
Gly (G)	Glycine
Glu (E)	Glutamic acid
H	Hydrogen
His (H)	Histidine
Hr	Hour

H_2O	Water
HBTU	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HCl	Hydrochloric acid
HOBT	Hydroxybenzotriazole
I	Iodo
I/R	Ischemia–reperfusion
IC_{50}	Inhibitory concentration
IDDM	Insulin dependent diabetes mellitus
IR	Infrared spectra
IS	Insertion sequences
*K*i	Inhibitor dissociation constant
L-chain	Long subunit chain
LC/MS	Liquid chromatography/Mass spectrometry
Leu (L)	Leucine
LGMD	Limb girdle muscular dystrophy
$LiAlH_4$	Lithium aluminium hydride
$LiBH_4$	Lithium borohydride
LTP	Long-term potentiation
M	Micromole per liter
MD	Muscular dystrophy
MeOH	Methanol
Met (M)	Methionine
$MgSO_4$	Magnesium sulfate
mM	Millimolar
MMMTS	Malignant mullerian mixed tumors
MS	Mass spectrometry
$NaHSO_3$	Sodium hydrogen sulfite
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
$Na_2S_2O_3$	Sodium thiosulphate
NCI	National cancer institute
nCL	Novel calpain large subunit
NIDDM	Non-insulin dependent diabetes mellitus
NIH	National institutes of health
Nle	Norleucine
NMDA	N-methyl D-aspartate
NMM	N-methyl morpholine
NMR	Nuclear magnetic resonance
$(o\text{-}Tol)_3P$	Tris(2-methylphenyl)phosphine
$Pd(OAc)_2$	Palladium(II) acetate
Phe (F)	Phenylalanine
PKC	Protein kinase C
PL	Phospholipase

Pro (P)	Proline
KCN	Potassium cyanide
RT	Room temperature
S-chain	Short subunit chain
SAR	Structure activity relationship
SCC	Squamous cell carcinoma
SEM	Standard error of mean
Ser (S)	Serine
SO_3	Sulfur trioxide
$SOCl_2$	Thionyl chloride
SRB	Sulforhodamine B
T1DM	Type I diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBI	Traumatic brain injury
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Thr (T)	Threonine
TLC	Thin layer chromatography
Tyr (Y)	Tyrosine
Val (V)	Valine

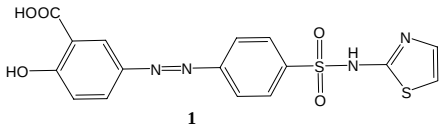
CHAPTER 1. INTRODUCTION

1.1 Research Objective

Calpain is a class of intracellular cytoplasmic cysteine proteases[1] that participates in different intracellular signaling pathways mediated by Ca^{2+}.[2] The precise in vivo function of calpain is not completely understood but the enzyme has attracted considerable attention because of its implication in a number of physiological and pathological conditions such as cancer, stroke, and Alzheimer's disease.[1, 3] Hence, potent and selective calpain inhibitors are attractive as therapeutic agents and as biomedical tools.[4]

Potent calpain inhibitors have been discovered in the past decade and the most potent inhibitors are peptidomimetic compounds such as MDL-28170[5-8] and SJA-6017,[9-12] which display calpain inhibitory activities in the nanomolar range. Nonpeptide calpain inhibitors have also been published but the calpain inhibitory activities of such compounds, unlike the peptidyl inhibitors, are in the micromolar range. Nonetheless, nonpeptide calpain inhibitors are still attractive because of their high selectivity and metabolic stability.

The problems of current calpain inhibitors are selectivity, cell permeability and metabolic stability. Several methods have been used to address the deficiencies. Some functional groups, such as benzyloxycarbonyl, sulfonyl, alkanoyl, substituted benzoyl, naphthoyl, xanthines,[13] and pyridineethanol,[9] have been used as N-terminal capping groups to improve potency, selectivity and solubility. Different α-ketone carbonyl groups have been incorporated at the P_1' position to explore activity.[14] Weakly basic groups have also been introduced at the P_1' position to increase solubility.[15] However, discovery of calpain inhibitors that incorporate potency, selectivity, metabolic stability, and adequate aqueous solubility within the same molecule is still a formidable challenge. Recent crystallographic data showing the active site structure of calpain with/without calpain inhibitors have made possible the development of potent and selective calpain inhibitors. Based on the X-ray crystal structure of the μ-calpain, virtual screening of the NCI compound library coupled with enzymological evaluation led to the identification of diazosulfonamide **1** (*K*i = 1.0 ± 0.02 μM, Fig. 1) as a new nonpeptide competitive inhibitor of μ-calpain. Sulfonamides have long been used as anti-infective agents in humans so this class of compounds is a good lead for optimization to afford novel inhibitors of μ-calpain.



1

Figure 1. Compound **1**.

Based on the identification of **1,** the objectives of this research were to (a) determine the structural requirements of diazosulfonamide **1** that are important for calpain inhibition as a prelude to the synthesis of novel sulfonamide-based peptidomimetic calpain inhibitors; (b) synthesize sulfonamide-based peptidomimetic calpain inhibitors; (c) assess the antiproliferative activity of the compounds since calpain has been implicated in carcinogenesis.

1.2 Overview of the Calpain Family of Cysteine Proteases

Calpain (EC 3.4.22.17)[16,17] is a member of the cysteine protease family, which has nucleophilic thiol group at active site. It is named because typical calpain possesses a calmodulin-like calcium-binding domain, "cal-", and a papain-like cysteine protease domain, "-pain".[18] Calpain was first observed in the soluble fraction of rat brain extracts by Guroff[19] in 1964 and requires Ca^{2+} for activity. Now crystal structures of calpain 1[20] and calpain 2[21-23] from human are available.

Numerous isoforms of calpain have been identified, widely distributed from mammals to invertebrate, fungi, yeast, and bacteria.[17] To date, sixteen different calpain isoforms have been identified in mammals[21,24] (Table 1) and could be classified into two groups, "typical" calpains and "atypical" calpains, based on whether or not they possess EF-hand motifs on the carboxyl terminal[24,25] (Figure 2). Two typical and ubiquitously distributed mammalian calpains are calpain 1 (μ-calpain), which requires micromolar concentration of calcium for activation in vitro, and calpain 2 (m-calpain), which requires millimolar concentrations of calcium.[18]

Calpain is also found in a wide variety of tissues, including skeletal muscle, cardiac muscle, brain, kidney, lung, liver, and adipose tissues.[26] The physiological or pathological regulation of calpain in these tissues is intricate and not well understood but it is acknowledged that calpain can cleave many intracellular signaling and structural proteins which play important roles in physiological or pathological conditions. Known calpain substrates include cytoskeletal and structural proteins (spectrin, microtubule-associated protein-2, tau factor, α-actinin, fodrin, dystrophin, tubulin), membrane bound receptors and proteins (EGF receptor, AMPA receptor, G-proteins, anion channel), calmodulin binding proteins (calcium pump, inositol 1,4,5-trisphosphate kinase), myofibrillar proteins (troponin I, troponin T, myosin), transcription factors (c-fos, c-jun) and some other important enzymes (protein kinase C, 3-hydroxy-3-methylglutaryl-CoA reductase, cAMP-dependent kinase).[27,28] The roles of the calpain substrates at pathological conditions have been studies but not well understood. But calpain has been implicated in muscular dystrophy, cataract, stroke, ischaemia, brain trauma, Alzheimer's disease, diabetes, and cancer.[27] As calpain is becoming an attractive therapeutic target, identification of calpain inhibitors is necessary.

Table 1. Human calpain expression profiles and diseases.

Name	Typical/Atypical	Tissue Expression	Diseases
Calpain 1	Typical	Ubiquitous	Huntington's disease, cataracts, stroke, muscular dystrophy, traumatic brain injury, spinal cord njury, alzheimer, cancer, multiple sclerosis, Lou Gehrig's disease, osteopenia
Calpain 2	Typical	Ubiquitous	Cataracts, muscular dystrophy, stroke, spinal cord injury, traumatic brain injury, Alzheimer, Parkinson, atherosclerosis, multiple sclerosis, Lou Gehrig's disease, cancer, psoriasis
Calpain 3	Typical	Skeletal muscle, lens, retina	Limb girdle muscular dystrophy 2A (LGMD2A), cataracts
Calpain 4	Typical	Ubiquitous	
Calpain 5	Atypical	Ubiquitous	Huntington's disease polycystic ovarian syndrome,metabolic syndrome
Calpain 6	Atypical	Placenta	
Calpain 7	Atypical	Ubiquitous	Huntington's disease
Calpain 8	Typical	Stomach mucosa	
Calpain 9	Typical	Digestive tract	Gastric cancer
Calpain 10	Atypical	Ubiquitous, and tissue specific isoforms	Huntington's disease, cataracts (?), diabetes mellitus, atherosclerosis, metabolic syndrome
Calpain 11	Typical	Testis	

Table 1 (continued).

Name	Typical/Atypical	Tissue Expression	Diseases
Calpain 12	Typical	Ubiquitous	Alzheimer (?)
Calpain 13	Typical	Testis/lung	
Calpain 14	Atypical	Ubiquitous	
Calpain 15	Atypical	Ubiquitous	CATM (hereditary cataract with microphthalmia) (?)
Calpain small subunit 2	Typical	N.D.	

N.D. –not determined

Sources:(1) Saez, M. E.; Ramirez-Lorca, R.; Moron, F. J.; Ruiz, A. The therapeutic potential of the calpain family: new aspects. *Drug Discov Today* 2006, *11*, 917-923.[24] Modified with permission from Elsevier Ltd. (2) Carragher, N. O. Calpain inhibition: a therapeutic strategy targeting multiple disease states. *Curr Pharm Des* 2006, *12*, 615-638.[25] Modified with permission from Bentham Science Publishers Ltd.

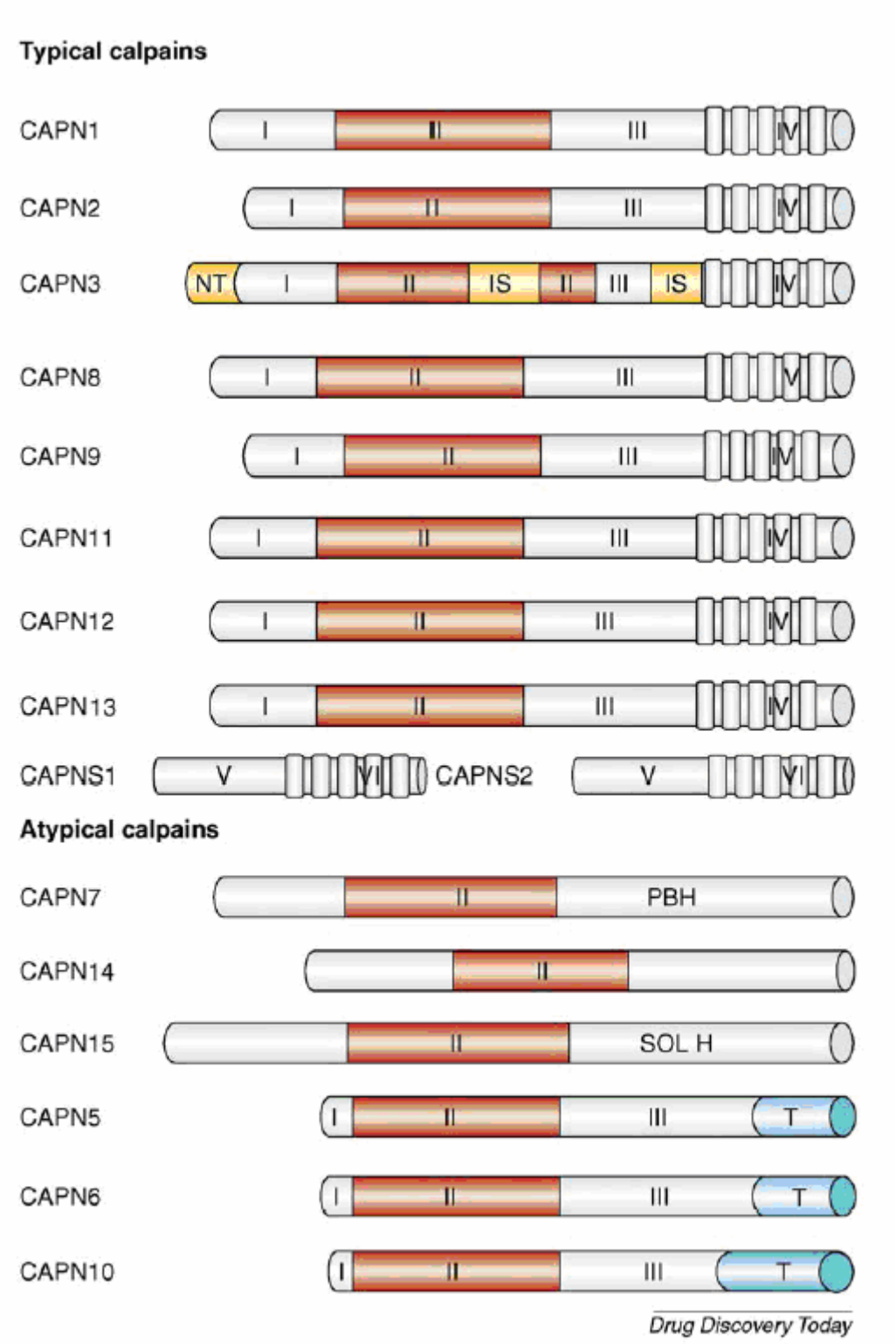


Figure 2. Schematic representation of the calpain family members.

Source: Saez, M. E.; Ramirez-Lorca, R.; Moron, F. J.; Ruiz, A. The therapeutic potential of the calpain family: new aspects. *Drug Discov Today* 2006, *11*, 917-923.[24] Adapted with permission from Elsevier Ltd.

It is important to note that calpain small subunit 2 was named as calpain14.[29] But after finding two new calpain genes, which are named as calpain13 and calpain14, old calpain13 was named as calpain15 and calpain small subunit 2 is not calpain14.[24,25] Not all calpain isoforms have been isolated at protein level, most of them have only been identified at the messenger ribonucleic acid (mRNA) level and only have predicted protein structure.[23]

1.2.1 Typical Calpains

Typical calpains, also named as EF-hand subfamily of calpains, include calpain 1, 2, 3, 4, 8, 9, 11, 12, 13 and calpain small subunit 2. They posses a Ca^{2+} binding EF-hand structure at the carboxy terminus.[25] EF-hand structure is a helix-loop-helix, calcium binding motif, in which two helices pack together at an angle of approximately 90 degrees, separated by a loop region, where calcium binds. The "EF" notation for the motif resulted from the structure of parvalbumin, in which the "E" and "F" helices were originally identified to form this calcium binding motif. [30]

1.2.1.1 Calpains 1 and 2

Calpain 1 and calpain 2, also known as μ-calpain and m-calpain, respectively, are the most extensively characterized members of the calpain superfamily. A large amount of calpain literature, including inhibitors, structure, and animal assays focus on these two isoforms. Both of them are ubiquitously distributed in mammalian cells. Calpain 1 requires micromolar concentrations of Ca^{2+} for activity in vitro whereas calpain 2 needs millimolar levels of Ca^{2+}. They function as hetero-dimers consisting of a similar large catalytic subunit and a common regulatory subunit (Figure 2). The large subunit is distinct for each calpain but the small subunit is identical. Calpain 1 has the large 82 kDa catalytic subunit and calpain 2 encodes the 80 kDa catalytic subunit. Both calpain 1 and calpain 2 associate with the small 28 kDa regulatory subunit. Calpain 1 and calpain 2 share approximately 60% sequence homology.[25]

The large subunit of calpain 1 and 2 can be sub-divided into four domains (I-IV) based on their amino-acid sequence.[25] Domain I (amino acids 1-87 in calpain 1 and 1-76 in calpain 2) is the N-terminal portion of the large subunit and is highly conserved between different species.[25] However, this region does not share any significant sequence homology with any other polypeptide and its precise function remains to be determined.[25] Domain I also contains the autolysis site and may be cleaved during the autolytic activation, which significantly increases sensitivity to Ca^{2+}.[21] Domain II (amino acids 88-327 in calpain 1 and 77-316 in calpain 2) is the catalytic protease domain which contains a catalytic triad (Cys-His-Asn) for substrate hydrolysis. The triad is composed of Cysteine (Cys), Histidine (His) and Asparagine (Asn) residues. Cys is located at residue 115 for human calpain 1 and 105 for human

calpain 2. The crystal structure of calpain 2 shows that domain II can be divided into two sub-domains (IIa and IIb) that are spatially separated in the absence of calcium.[22] The active site residue cysteine is located on subdomain IIa whereas histidine and asparagine are located on subdomain IIb.[25] Domain III (amino acids 328-569 in calpain 1 and 317-555 in calpain 2) is the linker between the catalytic domain II and the Ca^{2+}-binding domain IV. It does not have obvious sequence homology to any other protein[21]. But it has eight strands of β-sheets with a topology similar to the C2 domain, which is found in such proteins as protein kinase C and phospholipase C.[21] C2 domain can bind phospholipids in a Ca^{2+}-dependent manner and thus domain III is speculated to regulate calpain activity by involvement in electrostatic interactions with domain II.[25] Besides, when Ca^{2+} binds to domain IV, domain III changes its tertiary structure to increase the accessibility of the active site.[31] Domain IV (amino acids 570-714 in calpain 1 and 556-699 in calpain 2) is a calmodulin-like domain with five EF hand motifs. The first four EF hand motifs are thought to act as calcium binding sites while the fifth COOH-terminal EF-hand is involved in dimerization with the 28 kDa small subunit (calpain 4).[25]

1.2.1.2 Calpain 4 (Calpain Small Subunit 1, capns1)

Calpain 4 is well known as the small or regulatory subunit of calpains 1 and 2, and it is also known as calpain small subunit 1. It is composed of the NH_2-terminal domain V and the COOH-terminal domain VI.[32] Domain V (amino acids 715-781 in calpain 1 and 700-767 in calpain 2) is highly rich in glycine residues, containing about 30% glycine residues, and is referred to as a hydrophobic domain. The function of domain V has not yet been elaborated and it may act as a membrane anchor.[33] It is proposed that anchoring to membrane is a key step in the activation of the enzyme in vivo.[34,35] Domain VI (amino acids 782-984 in calpain 1 and 768-970 in calpain 2) is connected to domain V by a polyproline linker. It has about 50% sequence identity to domain IV and also contains five EF hand motifs, the first four of which bind calcium ions while the fifth can interact with its counterpart in another calpain molecule to form a homodimer or interact with the catalytic domain of calpain 1 or calpain 2.[25,36,37]

1.2.1.3 Calpain 3 (p94, nCL-1)

Calpain 3 gene (capn3), named as p94,[38] encodes an 821 amino acid protein,[39] which has different sequence with conventional calpain 1 and 2. mRNA expression of calpain 3 can be detected. However, calpain 3 protein is hardly detectable because it autolyzes rapidly after its translation with an estimated half-life of 27 minutes.[38] Calpain 3 is also known as novel calpain large subunit 1 (nCL-1), because its large subunit was different from calpain 1 and 2, and was the first discovered structure outside of the conventional calpain 1 and 2.[29]

Human calpain 3 shows significant sequence homology with the large subunit of human calpain 1 and 2, 54% and 51% respectively,[40] and can also be divided into four similar domains. As calpain 1 and 2, domain II contains the active site Cys, His, Asn residues necessary for catalysis. But unlike calpains 1 and 2, calmodulin-like calcium-binding domain IV[40] does not bind nor interact with domain VI of calpain 4.[2]

Besides, calpain 3 is over 100 amino acid residues longer and has three unique sequences (NS, IS1 and IS2) compared to the conventional calpain. NS, which is at the N-terminal specific region of domain I, is about 60 residues in length and is rich in proline (P) residues at the amino terminal end.[41] The precise function has not yet been clarified but it is believed to play an important role in the overall tertiary structure of the enzyme.[2] IS1, located in the middle of domain II, has 62 amino acid residues.[38] Its function has not been elucidated, but may be involved in regulatory activity.[42,43] IS2, located at the C-terminal of domain III, has 77 amino acid residues.[38] It is rich in lysine residues at the amino terminal end and possesses a nuclear localization-like sequence, which suggests that calpain 3 could play a role in nuclear localization.[41]

In muscle, mRNA expression of calpain 3 is at least 10 fold higher than that of calpain 1 and 2.[38,40,41] A disruption of the calpain 3 gene resulted in limb girdle muscular dystrophy type 2A (LGMD2A), suggesting that the protein may have a specific role in the physiological functions of the skeletal muscle.[44] Three splice variants of calpain 3, Lp82, Lp85, and Rt88, have been detected in rat visual system. These variants of calpain 3 are believed to be involved in lens development and maturation.[45-47]

1.2.1.4 Calpain 8

Calpain 8, also known as nCL-2 (novel calpain large subunit 2), was found by searching rat cDNA libraries of various tissues.[29] The mRNA level of calpain 8 is predominantly expressed in the stomach, and weak expression is observed in the small intestine,[2,48] heart and skeletal muscle.[2] Like calpain 3, calpain 8 does not associate with the small regulatory subunit.[49] But calpain 8 does not have the NS, IS1, and IS2 sequences which is specific for calpain 3. Calpain 8 has high sequence homology to the domain structure of conventional calpain.[50] The large subunit of calpain 8 is 58% homologous to human calpain 1 and 61% homologous to calpain 2.[51] Calpain 8 contains 703 amino acid residues and can also be divided into four domains. Domain II, which has over 70% similarity to m- and μ-calpains, is the most conserved. Domain IV has four EF-hand structures.

Calpain 8 can be alternatively splice to give nCL-2', which contains 381 amino acid residues. nCL-2' has identical large subunit as nCL-2 but two thirds of domain III and calcium binding domain IV are missing.[29] Therefore, nCL-2' is assigned to be

a member of the non-EF hand subfamily. The mRNA level of both nCL-2 and nCL-2' expressed in the stomach are almost equal with calpain 1 and 2. This suggests that nCL-2 and nCL-2' may play an important role in the stomach as conventional calpains 1 and 2 in the stomach.[48]

1.2.1.5 Calpain 9

Calpain 9, also known as nCL-4 (novel calpain large subunit 4), was identified by the screening of rat cDNA. It comprises 690 amino acids and is expressed predominantly in the digestive organs such as stomach and small intestine, and in the uterus.[2] It has a total sequence identity of 54% with human calpain 1, 51% with human calpain 2, 52% with rat calpain 8, and 55% with human calpain 3 when the NS, IS1, and IS2 regions are excluded.[48] The expression of calpain **9** is downregulated in gastric cancer tissues and cell lines.[52] The depletion of calpain 9 by antisense RNA results in cell transformation and tumourigenesis in murine NIH 3T3 fibroblasts.[53,54] These indicate that calpain 9 may function as a tumour suppressor.

1.2.1.6 Calpain 11

Calpain 11 was discovered by a search of the protein sequences in the commercially available INCYTE EST database. The predicted calpain 11 protein is a 702 amino acid protein and has a total sequence identity of 58% with chicken μ/m-calpain and 54% with human calpain 1.[55] Domain II of calpain 11 contains C, H, and N amino acids to be a catalytic triad (Cys-His-Asn). Dmain IV has five calcium-binding sequences which suggest that calpain 11 could also possess calcium-binding ability.[55] mRNA analysis of different tissues suggested that the highest level of Capn11 is presented in spermatocytes during the later stages of meiosis.[55,56] Testis-specific calpain 11 could be involved with testis-specific transcription factor regulation or germ cell apoptosis.[55]

1.2.1.7 Calpain12

Calpain 12 was discovered by searching the GenBank mouse EST database with protein sequences of known vertebrate calpains.[57] Calpain 12 has 720 amino acids and predicted sequence has a homology of 40% with calpain 2.[57] I t can also be divided into four domains (I-IV), similar to conventional calpains. Calpain 12 has catalytic domain II and a Ca^{2+}-binding domain IV. Domain II contains C, H, and N amino acids to be catalytic triad and domain IV has five calcium-binding sequences. RT-PCR analysis has shown that the CAPN12 gene is ubiquitously expressed but the highest amounts are found in cortex of the hair follicles.[57]

1.2.1.8 Calpain13

Calpain 13 is predominantly distributed in testis, like calpain11, and has also been detected in the lung.[58] The functions and their eventual role in human diseases remain unexplored.[24]

1.2.1.9 Calpain Small Subunit 2 (capns2)

Calpain small subunit 2 is highly homologous (63%) to small subunit 1 with fewer glycine residues in its domain V than small subunit 2.[29] Its function is unclear.[17,24]

1.2.2 Atypical Calpains

"Atypical" calpains (calpain 5, 6, 7, 10, 14 and15) do not possess a domain IV that contains EF-hand calcium binding sites at their carboxy terminus. It is unclear whether the atypical calpains require calcium for activity.

1.2.2.1 Calpain 5 (nCL-3, htra-3)

Calpain 5 (nCL-3, or htra-3) was identified by a search of the GenBank database with the amino acid sequence of domain II of human calpain 1.[59] Calpain 5 is named as nCL-3[29] because it was the third novel large subunit identified. Calpain 5 is considered as htra-3 because it is the human homologue of tra-3. Domain I-III of calpain 5 has 35% similarity with the tra-3, which is a sex determination gene of the nematode.[59]

The predicted 634 amino acid sequence of calpain 5 has similar large subunits with calpains 1 and 2 from domain I to III and has a novel region termed domain T. Domain T does not have the conventional EF-hand motifs for Ca^{2+}-binding as found in domain IV.[2] It is unclear whether calpain 5 is Ca^{2+}-dependent.

The mRNA of calpain 5 is present in many tissues, but its highest expression is in the colon, small intestine, and testes.[29] Since the calpain 5 gene is required for correct sexual development in hermaphrodites (e.g., nematodes), it is thought that calpain 5 plays a role in sex determination and development in humans.[59]

1.2.2.2 Calpain 6

Calpain 6 was identified by a search of the GenBank database with the amino acid sequence of domain II of human calpains 1 and 2.[17] It is also known as CAPNX because of its location on the X chromosome.

The predicted amino acid sequence of calpain 6 has a similar domain II with calpains 1 and 2. The cDNA of calpain 6 shows that it does not have the active site cysteine residue but rather a nonfunctional lysine residue.[29] As a result, the protein of calpain 6 is predicted to be protolytically inactive.[59] The same thing was observed in mice. In addition to the absence of cysteine residue, the mouse mRNA of calpain 6 does not translate an active site His residue in domain II either.[49] Calpain 6 has 47% homology to calpain 5.[17,60] Both calpain 6 and calpain 5 lack the Ca^{2+} binding domain IV, and is replaced with a T-domain. The function of domain T is still unknown.

The physiological and pathological roles of calpain 6 remain to be determined. mRNA of calpain 6 is expressed in skeletal, heart muscles and in specific cells of the lung, kidney and various epithelial cell types.[56] Out of 50 tissues tested, the highest level of calpain 6 mRNA was expressed only in the placenta, which suggests that the protein could play a role in the development process.[29] Calpain 6 is also speculated to play a role in sex determination and development because of its similarity to tra3 and calpain 5, and due to its location on human X chromosome.[29]

1.2.2.3 Calpain 7 (PalBH)

Calpain 7 was identified by a search of the EST database at the National Center for Biotechnology Information with nucleic acid and amino acid sequences of known vertebrate calpains. Its predicted 813 amino acid protein product[61] shares 26-35% identity to the rest of the calpain members.[17,29,61] and has little homology in domains III and I of calpain 1 and 2.[29] Domain II of calpain 7, which contains the active site C, H, and N residues, is similar with conventional calpains (calpain 1 and 2).[61,62] The carboxyl terminal domain shows no homology to either the calmodulin-like domain IV or to domain T of calpains 5 and 6[61] but its carboxyl terminal end is homologous to the PalB fungal protease that is involved in alkaline ambient pH adaptation in the fungus *Aspergillus nidulans*.[29] So calpain 7 is also known as the PalBH (PalB homologous domain).

Calpain 7 appears to be ubiquitously distributed but its exact function is unknown. The levels of calpain 7, along with other calpain family members, such as calpain 1, 5 and 10, are increased in Huntington's disease target-tissue culture and transgenic mouse models, suggesting that they might contribute to this neurological pathology.[24]

1.2.2.4 Calpain 10

Calpain 10 was discovered and cloned during a search aimed at identifying type 2 diabetes susceptibility genes.[60] The predicted protein sequence contains the catalytic triad (C, H, and N) at active site. The C-terminal of calpain 10 does not have EF-hand motifs as typical calpains and is homologous to domain T in calpain 5 and 6.

High mRNA levels of calpain 10 are found in heart, pancreas, brain, liver, and kidney. Calpain 10 is thought to play a role in type 2 diabetes.[63-66]

1.2.2.5 Calpain 14

Calpain14 was reported on 2001 with calpain 13.[58] However, the mRNA of calpain 14 could not be detected in any of the 76 tissues examined. Radiation hybrid mapping localized the gene within a region mapped to 2p21-2p22.[58]

1.2.2.6 Calpain 15 (Sol H)

A search of the human EST database showed that calpain 15 has 44% sequence homologue[67] with sol, the embryo-specific Drosophila small optic lobe gene product[29] hence the ubiquitously distributed human calpain 15 is sometimes referred as SolH.[29]

The predicted 1086 amino acid protein contains Cys, His, and Asn active site residues within a similar domain II region.[29] Like Sol, predicted calpain 15 possesses five copies of a zinc-finger-like motif in its amino terminal portion[67] instead of calmodulin-like domain IV.

Calpain 15 is expressed at low levels in most tissues. its role has not yet been fully determined, but calpain 15 gene has been implicated in a chromosomal translocation in a family affected by hereditary cataract with microphthalmia (CATM).[24,68]

1.3 Calpain and Disease

1.3.1 Limb-Girdle Muscular Dystrophy 2A (LGMD2A)

In 1954, Walton and Nattrass established limb-girdle muscular dystrophy (LGMD) as an entity distinct from other muscular dystrophies.[69,70] In 1995, the European Neuromuscular Centre Workshop established more precise criteria for diagnosis and classification of LGMD, grouping the different subtypes according to

their genetic characteristics.[69] The designation for autosomal-dominant limb-girdle dystrophies is now LGMD1, whereas autosomal-recessive forms are LGMD2.[69] Numerous reviews and individual reports suggest that autosomal recessive is much more common than autosomal dominant LGMD. Among patients with LGMD2, types 2A appears to be one of the more common subtypes.[69] LGMD2A is an autosomal recessive form of the disease in which the muscle specific calpain 3 (also known as p94) is the primary cause.[44]

The onset of LGMD2A is usually in the second decade of life, although it has been documented to occur as early as age 2.5 and as late as age 49.[71] The course of the disease is slow but progressive, leading to loss of ambulation by approximately 1 or 2 decades after diagnosis. Scapular winging and hip abductor sparing are two common features.[71] At the current time, no effective treatment is available to patients with LGMD.[69]

Inactivating mutations in the calpain 3 gene are responsible for limb-girdle muscular dystrophy LGMD2A.[72] Genetic quest for the etiologic gene of a cluster of LGMD patients in the southern part of the isle of La Réunion led to the initial mapping of the disease locus to the gene CAPN3, encoding the calpain 3 protease.[71] There are now over 280 documented distinct pathogenic CAPN3 mutations. These CAPN3 mutations span almost the entire length of the CAPN3 gene with no major predominant mutations.[71] Of interest is the fact that numerous mutations lead to loss of calpain 3 protein, indicating that lack of this protease may lead to pathogenic consequences.[71]

The exact mechanisms involved in the pathogenesis of LGMD2A are still unknown. Since calpain 3 is a protease with a potentially broad range of substrates, there is a high probability that it is involved in regulating multiple physiological processes. This diverse role for calpain 3 means that its absence or mutations in CAPN3 can affect many pathways in muscle cells.[71] Hypothetical mechanism (Figure 3) was proposed to explain why the loss of proteolytic capability of calpain 3 might lead to cell dysfunction. Mutations in CAPN3 can lead to loss of proteolytic activity or loss of titin anchorage. The latter is likely to reduce regulation of proteolytic activity or to remove calpain 3 from its substrates. Loss of proteolysis of substrates can cause accumulation of proteins that can subsequently be damaged and then aggregate. These aggregates can impair proper muscle function and impair growth, leading to muscular dystrophy.

1.3.2 Diabetes

Diabetes mellitus is a metabolic disease which is characterized by high blood glucose levels because the body does not release or use insulin adequately. If untreated, it can lead to blindness, kidney and heart disease, stroke, loss of limbs and

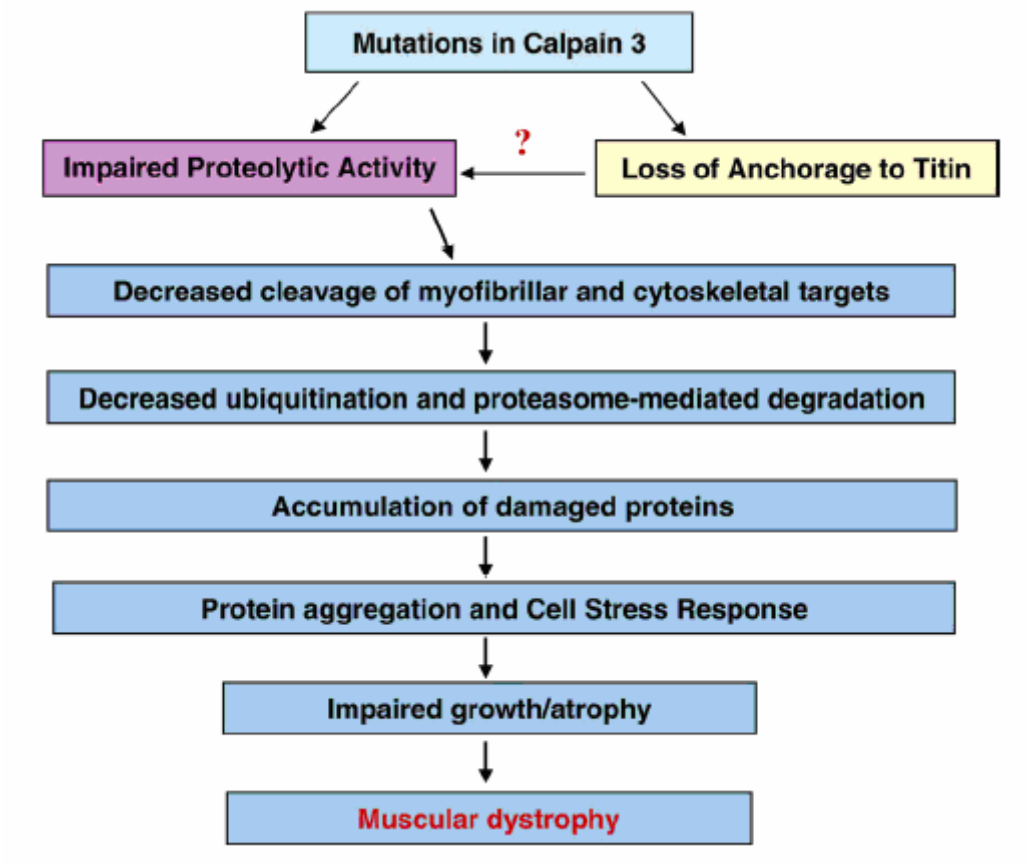


Figure 3. Flow chart demonstrating the pathogenic consequences of calpain 3 deficiency. Mutations in calpain 3 can lead to loss of proteolytic activity or loss of titin anchorage. The latter is likely to reduce regulation of proteolytic activity or to remove calpain 3 from its substrates. Loss of proteolysis of substrates can cause accumulation of proteins that can subsequently become damaged and then aggregate. These aggregates can impair proper muscle function and impair growth, leading to muscular dystrophy.

Source: Kramerova, I.; Beckmann, J. S.; Spencer, M. Molecular and cellular basis of calpainopathy (limb girdle muscular dystrophy type 2A). *Biochim Biophys Acta* 2007, *1772*, 128-144.[71] Adapted with permission from Elsevier Ltd.

diabetes, is caused by autoimmune destruction of the β cells of the pancreas, reduced life expectancy.[60] Type I diabetes mellitus (T1DM), or insulin-dependent insulin-producing cells, rendering the pancreas unable to synthesize and secrete insulin.[73-76] T1DM accounts for approximately 5–10% of all cases of diabetes with the major susceptibility gene mapping to the HLA region of chromosome 6.[73,77] Type 2 diabetes mellitus (T2DM), or non-insulin-dependent diabetes mellitus (NIDDM), which is characterized by defects in hepatic glucose production, insulin action and insulin secretion,[60,73] is the most common form of diabetes. T2DM accounts for approximately 90% of cases and affecting 10–20% of those over 45 years of age in many developed countries.[60] It has been predicted that T2DM will affect 215 million people worldwide by 2010.[73,77]

For both TIDM and T2DM, in most cases, the precise biochemical defects, genetic causes and other contributing factors of these diseases are not fully elucidated.[73] However, it is becoming increasingly clear that members of the calpain family are involved in T2DM.[73,77]

In 1996, genome-wide scan studies for type 2 diabetes genes carried out in Mexican Americans localized a susceptibility gene, designated NIDDM1, at 2q37.3.[60,78] Four years later, positional cloning studies revealed that NIDDM1 was a single gene, CAPN10, which encodes the intracellular cysteine protease, calpain 10.[77] This is the first diabetes gene identified through a genome scan.[79] More recently attention has started to shift to focus on the functional biology of calpain-10 in pancreatic β-cells, skeletal muscle and adipocytes.[79]

A number of studies have suggested that calpains may be involved in T2DM-related pathways.[77] Turner described the putative role(s) of calpain 10 in T2DM pathways in Figure 4.[79]

Knowledge about the role of calpain 10 in diabetes will lead to improved diagnosis, treatment and prevention, and thereby could help to reverse the predicted rise in the prevalence of this disorder.[60]

1.3.3 Cataract

Cataract is a clouding of the eye's naturally clear lens.[80] According to the World Health Organization, cataract is the leading cause of blindness in the world and affects nearly 20.5 million Americans age 40 and older.[80,81] The exact cause of cataract is unclear, but it may be the result of a lifetime of exposure to ultraviolet radiation contained in sunlight, or may be related to other lifestyle factors such as cigarette smoking, diet, and alcohol consumption.[81,82] To date, cataract removal is the most common treatment and more than a million such surgeries are performed each year[80,82]

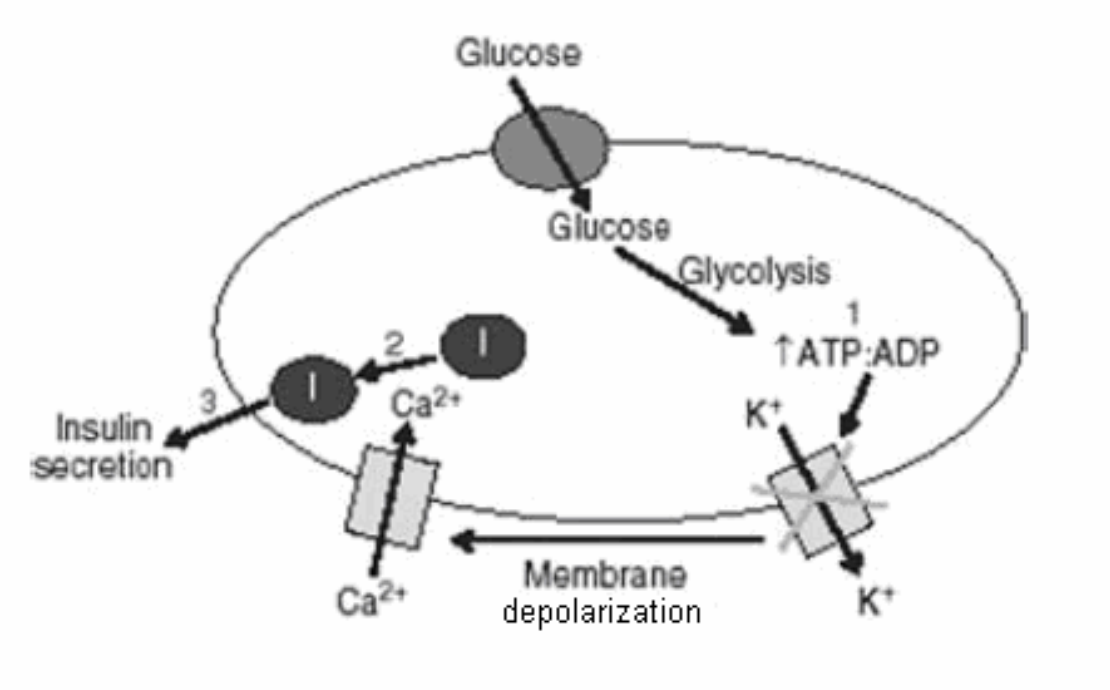


Figure 4. Proposed sites of action for calpain 10 in T2DM-related pathways. (1) The internalization of glucose leads to glycolysis and elevated ATP:ADP ratios through the action of mitochondria. This action is strongly diminished by calpain inhibition and calpain 10 is suggested to act as a regulator of mitochondrial fuel sensing. (2) The secretory stimulus is accompanied by cytoskeletal rearrangement, which leads to the transport of secretory granules containing insulin to active sites of exocytosis at the plasma membrane. In the case of adipocytes and skeletal muscle, this process proceeds via the translocation of vesicles containing GLUT4. This process is highly sensitive to calpin inhibition, either through the use of inhibitors or specific CAPN10 antisense nucleotides, and roles for calpain 10 and calpain 1 have been suggested. (3) Exocytosis is mediated by a SNARE family of proteins and a specific isoform of calpain 10 has been shown to associate with this family. Based on a number of evidence, calpain-10 has been proposed to be a pivotal Ca2+-sensor in exocytosis in β cells.

Source: Harris, F.; Biswas, S.; Singh, J.; Dennison, S.; Phoenix, D. A. Calpains and their multiple roles in diabetes mellitus. *Ann N Y Acad Sci* 2006, *1084*, 452-480.[77] Adapted with permission from New York Academy of Sciences.

However, surgery is not truly a cure for cataract [83] and the cost of cataract surgery limits individuals in many parts of the world. So there is an urgent need for less expensive, nonsurgical approaches to cataract treatment.[83] Now there are no therapeutic agents that can significantly prevent and treat cataract.

Biochemists are analyzing the formation of cataracts at a molecular level and five calpains are known to occur in the lens, including calpain 1,[29] Lp85,[84] calpain 2,[85] calpain 10[86] and Lp82, a lens-specific splice variant of calpain 3.[83] If calpain inhibitors can be used as anticataractogenic agents, they could be economical nonsurgical agents for cataract treatment. Some evidences suggest that calpain inhibitors would be beneficial in prevention and treatment of cataract. E64 showed some ability to reduce cataract in rodent models.[87] E64d, derivative of E64, also prevented induced cataract in lens culture models.[88,89] SJA6017, a high selective calpain inhibitor, was found to be able to prevent both Ca^{2+}-induced nuclear opacity and crystallin proteolysis in a way that correlated with its ability to inhibit calpain 2.[10,11,83] More recently, SJA6017 has been shown to retard calpain-mediated cataractogenesis in porcine lenses[90] and Lp82-mediated cataractogenesis in rodent lenses.[83]

1.3.4 Cancer

Association between abnormal calpain activity and tumorigenesis has been observed in many studies. Many gene products of oncogenes and tumor suppressor genes are substrates of calpain family enzymes, including c-fos, c-jun, p53, pp60src, the estrogen receptor and the adhesion molecule integrin.[29] Activities of calpain were significantly higher in breast cancer tissues, compared with those of normal breast tissues[54], and were higher in the ER (estrogen receptor)-positive tumors than in ER-negative ones.[25,29,91] Treatment of ER positive breast cancer cells with the synthetic peptide calpain inhibitor calpeptin can inhibit the growth of ER positive breast cancer cells but had no effect on growth of ER negative breast cancer cells.[25,92] Calpain mediated cleavage of NF2 has been proposed as a mechanism that initiates tumourigenesis in a subset of schwannomas and meningiomas.[93] Calpain also plays a positive regulatory role in melanogenesis through modulating the expression of tyrosinase.[94] The endogenous calpain inhibitor was selectively cytotoxic to human tumor cells from lung, bladder, melanoma and chronic myeloid leukemia tissues, in a dose-dependent manner, but not cytotoxic to normal human, urothelial, fallopian tube, liver and resting white blood cells.[95] The inhibitor was also tested in vivo on Wistar rats bearing Walker tumors. Treatment with 50 Units/100 g i.p. daily for 5 days caused 90% tumor regression and necrosis of metastatic foci in the liver and abdomen, without toxic side effects.[95]

At the molecular level, calpains 1, 2, 6 and 9 are thought to play a role in carcinogenesis. High expression levels of calpain 1 mRNA in tissue samples from human renal cell carcinoma correlated with metastasis to peripheral lymph nodes

suggesting a role for calpain in both carcinogenesis and tumour progression.[25,91,96] Calpain 1 mRNA levels, were also found to be significantly increased in basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) when compared with normal human skin.[25,97] In chronic lymphocytic leukaemia (B-CLL) cells, calpain 1 activity was found to be significantly elevated when compared with non-malignant cells.[25,98] Antisense-mediated suppression of calpain 2 gene expression reduced the invasion of human DU 145 prostate carcinoma cells both in vitro and in vivo.[25,99] This study provides the first direct evidence that intervention against a single calpain isoform can influence tumor cell progression.

It has been suggested that calpain 6 may be associated with the development of uterine sarcoma and uterine malignant mullerian mixed tumors (MMMTs). Although the precise effect of increased expression of capn6 need further investigation, but calpain 6 may play a role as a novel tumor marker in those diseases.[100]

Expression of calpain 9 was downregulated in gastric cancer tissues and cell lines[25,52] and the depletion of calpain 9 by antisense RNA strategy resulted in cell transformation and tumourigenesis in murine NIH 3T3 fibroblasts.[25,53,54] These results suggest that calpain 9 might be a new type of tumor suppressor. Thus, identification of endogenous substrate(s) of calpain 9 might help to define underlying mechanisms in the development of gastric cancer.

1.3.5 Alzheimer's Disease

Alzheimer's disease (AD) is a brain disorder that seriously affects a person's ability to carry out daily activities, especially among older people. This disease usually begins after age 60, and risk goes up with age. About 5 percent of men and women ages 65 to 74 have AD, and nearly half of those age 85 and older may have the disease. While younger people also may get AD, it is much less common. As many as 4.5 million Americans suffer from AD, according to the data published by NIH in 2006.[101] The etiology of AD is not fully clear. There probably are several factors that affect each person differently. Age is the most important known risk factor for AD.[101]

Molecular studies have shown that calpain is involved in AD. The two typical brain lesions in Alzheimer's disease are extracellular amyloid plaques, which are deposits of β-amyloid peptides, and intracellular neurofibrillar tangles, which are composed primarily of the hyperphosphorylated microtubule-associated tau protein.[17,78] β-Amyloid peptides, the major components of amyloid plaques in the brains of AD patients,[102] can activate calpains, thereby mediating the cleavage of PARP[103], an enzyme that is thought to contribute to the pathophysiology of AD by inducing neuronal apoptosis.[102,104] Calpain is also thought to cleave p35,[105] a regulator of cyclin-dependent kinase 5 (cdk5) in the CNS, into two proteins, p10 and p25, and then the latter activates cdk5.[104-106] p25/cdk5 can hyperphosphorylate tau, a substrate

for CDK5. The hyperphosphorylated tau protein results in the formation of neurofibrillary tangles in AD's brains.[78,104]

Both m-calpain and μ-calpain may be involved in Alzheimer's disease.[107,108] Activated form of m-calpain is increased in the brains of AD patients.[109,110] μ-Calpain is abnormally activated in AD brain[108] and calpastatin, the endogenous inhibitor of calpains, is significantly decreased.[109,111] These observations suggest the hypothesis that inhibition of calpain activity should prevent or retard the evolution of AD.[109]

1.3.6 Ischemia

Ischemia is an absolute or relative shortage of the blood supply to an organ. Calpain is suspected to play a major role in cerebral ischemia (stroke), traumatic brain injury (TBI),[29,112] and ischemia in heart,[27] kidney,[113,114] and liver.[115]

Cerebral ischemia is an ischemic condition where the brain or parts of the brain do not receive enough blood flow to maintain normal neurological function. Traumatic brain injury (TBI), also called intracranial injury, or simply head injury, occurs when a sudden trauma causes brain damage. Cerebral ischemia and traumatic brain injury (TBI) represent the two most common and well-studied manifestations of in vivo excitotoxicity. In cerebral ischemia and traumatic brain injury, decreased blood flow in brain areas results in increases in presynaptic vesicular glutamate release and inhibition of glutamate re-uptake by adjacent astrocytes (Figure 5).[29] The resultant excessive glutamate overactivates ionotropic glutamate receptors (NMDA, AMPA and kainate receptors),[29] which allows Na^+ and Ca^{2+} influx, in the postsynaptic membrane. The elevated intracellular Na^+ level further triggers the opening of voltage-gated neuronal Ca^{2+} channels, and the resultant Ca^{2+} overload leads to activation of several calcium-dependent enzymes, including protein kinase C (PKC), calmodulin (CaM)-dependent NO synthase (NOS), protein kinase II (CaMPK-II), phospholipase A2 (PLA2), and especially calpain 1 and 2.[29,87] A number of subcellular targets have been identified as substrates for calpain cleavage in the brain, including cytoskeletal proteins, membrane proteins and various other proteins,[28] but the precise physiological role of calpain is not yet clearly understood. It is acknowledged that overactivated calpain could lead to uncontrolled degradation of cytoskeletal proteins, cytosolic and nuclear enzymes and, ultimately, neuronal death.[29] Calpain 1 and 2 play a role in disabling the neurons in signal transduction, membrane and cytoskeleton integrity and nuclear function.[29] Calpain inhibitor I and leupeptin can reduce neuronal damage in global ischaemia.[116] Calpain inhibitor I and MDL 28170 can improve neuronal recovery from hypoxia.[117,118] MDL 28170 also can reduce infarct size even 6 hours after the initiation of ischemia in a rat model of focal cerebral ischaemia, showing that calpain activation may be an obligatory, downstream event in the ischemic cell death cascade.[119-121] Calpain inhibitors AK295 and AK275 provide significant neuroprotection in ischemia-induced proteolysis.[122,123] All these

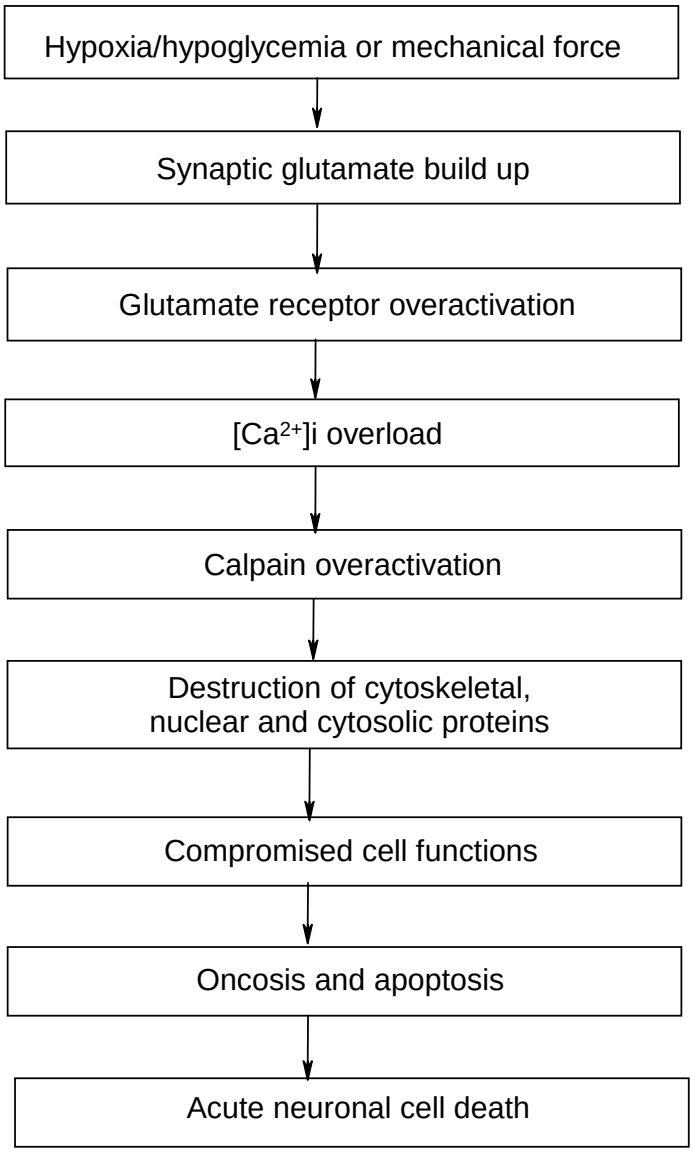


Figure 5. Calpain 1 and 2 in cerebral ischemia and traumatic brain injury.

Source: Huang, Y.; Wang, K. K. The calpain family and human disease. *Trends Mol Med* 2001, *7*, 355-362.[29] Modified with permission from Elsevier Ltd.

cases indicate that inhibition of calpain may be a feasible therapy to maintain neuron functions.[121]

Myocardial infarction is a medical condition that occurs when the blood supply to a part of the heart is interrupted. The resulting ischemia or oxygen shortage causes damage and potential death of heart tissue. Calpain is activated during myocardial ischaemia and calpain inhibitors showed beneficial effects. However, this area is not yet clearly understood. E64c was used as a potential protective agent in a dog model where acute myocardial infarction was induced by occlusion of the left anterior descending artery. The combination of E64c and reperfusion was found to significantly reduce infarct size.[87]

Ischemia–reperfusion (I/R) injury involves a cascade of events that ultimately lead to cellular damage and organ dysfunction. It is one of the most important issues to be resolved in liver surgery. The activation of calpain appears essential in I/R-induced apoptosis, but cross-talk between calpain and the caspase systems in apoptosis remains unclear at present. In addition to calpain and caspases, other proteinases such as nuclear serine protease and cathepsin B may also contribute to the apoptosis of hepatocytes. In a rat model of hepatic I/R injury, the calpain inhibitor, Cbz-Val-Phe methyl ester, inhibited cell necrosis and apoptosis of the sinusoidal endothelial cells and hepatocytes, suggesting that calpain may play a role upstream of caspases.[124]

1.4 Calpain Inhibitors

Crystal structures of calpain with inhibitor proved that calpain inhibitors can target against the active catalytic site or interact with other allosteric sites of calpain that are important in catalytic activation.[13,125] Based on different binding position, calpain inhibitors can be divided into active site directed calpain inhibitors and allosteric inhibitors.

1.4.1 Active Site Directed Calpain Inhibitors

Active site directed calpain inhibitors usually are the peptidomimetic of calpain substrate. They are composed of an address region for enzyme recognition and a "warhead" for interaction with the active site cysteine residue in enzyme (Figure 6). The warhead is an electrophilic group which can react either reversibly or irreversibly with the thiol in the cysteine residue of calpain. According to the different interaction with the cysteine residue of calpain active site, active site directed calpain inhibitors can be sub-divided into irreversible and reversible calpain inhibitors.

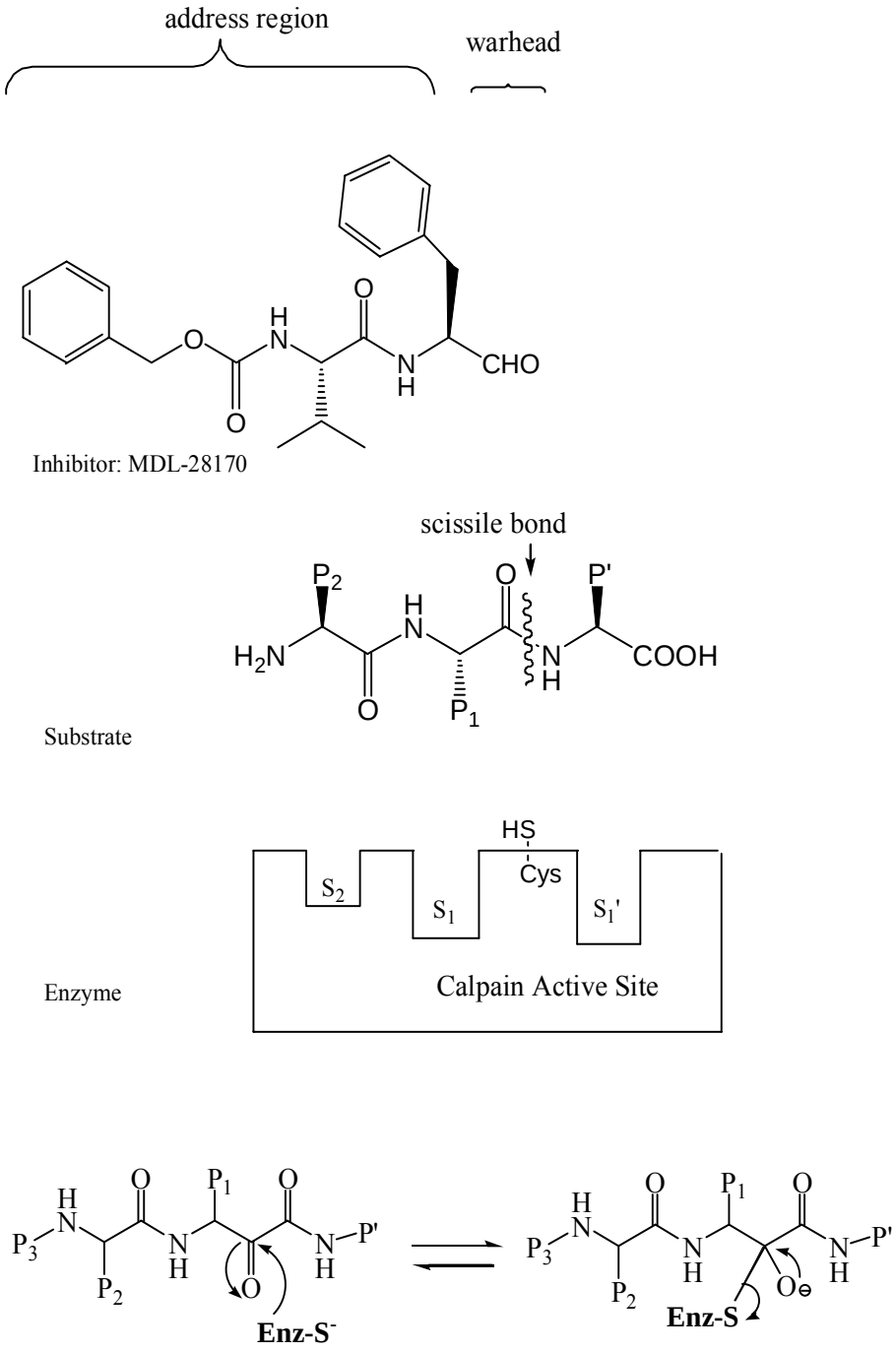


Figure 6. Relationship among calpain, substrate and inhibitors. Scissile bond refers to the bond in the substrate that is hydrolyzed by the enzyme. The address region of calpain inhibitors is region for enzyme recognition. P_1, P_2 and P_3 refer to the side chain of different amino acid from the scissile bond to the amino terminal. P' refers to the side chain of different amino acid from the scissile bond to the acid terminal. S_1, S_2 and S_2 refer to the binding positions of enzyme in the corresponding to P_1, P_2 and P' positions of the peptide substrate.

1.4.1.1 Irreversible Calpain Inhibitors

Peptidyl epoxysuccinate calpain inhibitors are a well-studied group of irreversible calpain inhibitors. Epoxysuccinyl derivate E-64 (Figure 7), isolated from Aspergillus japonicus,[25] is used in many publications. It is selective for cysteine proteases[13] but not cell permeable because of the presence of charged carboxylate and guanidinium groups.[25] Modification of E64 by esterification of the carboxyl group and modification of the guanidinium group to a benzyloxycarbonyl group resulted in a cell permeable compound E64d (Figure 7), which has an N-terminal capping with a benzyloxycarbonyl group, and can be converted in vivo to its active form E64c (Figure 7) by hydrolysis of the ester.[25,126] Intraperitoneal injection of E64 prior to selenite induced cataract formation in mice can reduce both the frequency and severity of cataract formation.[25,127] Administration of E64d in vivo can reduce muscle degeneration and muscular dystrophy in dystrophic mice and hamster models.[25] Also a significant neuroprotection by E64d following spinal cord injury in rats has been reported.[25,128] E64c was reported to significantly reduce ischaemic injury and infarct size in a canine model of acute myocardial infarction.[25,129] In cultured lenses, E64, E64c and E64d can reduce nuclear opacity by calcium ionophore A23187 in a concentration-dependent manner, and E64d ,was the most effective.[89]

Besides epoxide group, reported warhead replacements of irreversible calpain inhibitors are ketomethylenes, methylsulfonium salts, vinyl sulfones, and disulfide linkages (Figure 8). Recently published potent selective compounds don't show these functional groups and focused on reversible calpain inhibitors. Irreversible calpain inhibitors have high activated electrophilic groups which is difficult to differentiate thiol in cysteine protease with other nucleophilic groups in protease (for example, hydroxyl group in serine protease) and cause unexpected adverse effects.

1.4.1.2 Reversible Calpain Inhibitors

Reversible calpain inhibitors are derived from natural sources, such as calpastatin and leupeptin, and chemical synthesis sources. Functional groups have been reported to be warheads are aldehydes, α- ketocarbonyls, and α-keto heterocycles in reversible calpain inhibitors. The α-ketocarbonyl compounds include α-ketoacids, α-ketoamides,[130] α-ketoesters,[130] α-diketones, and α-ketophosphorous[131] (Figure 8). Because aldehyde is more electrophilicity, less electrophilic α-keto carbonyl compounds are more possible to be selective to calpain over other cystein protease. So recent published potent active site directed reversible calpain inhibitors are focused on peptidyl aldehydes and peptidyl α-ketoamides.[25] Besides, replacing the metabolically labile aldehyde moiety with cyclic hemiacetal, for example SNJ-1715 (Figure 7), could increase potency, aqueous solubility, excellent oral bioavailability, and prolonged plasma half-life in rat model.[132]

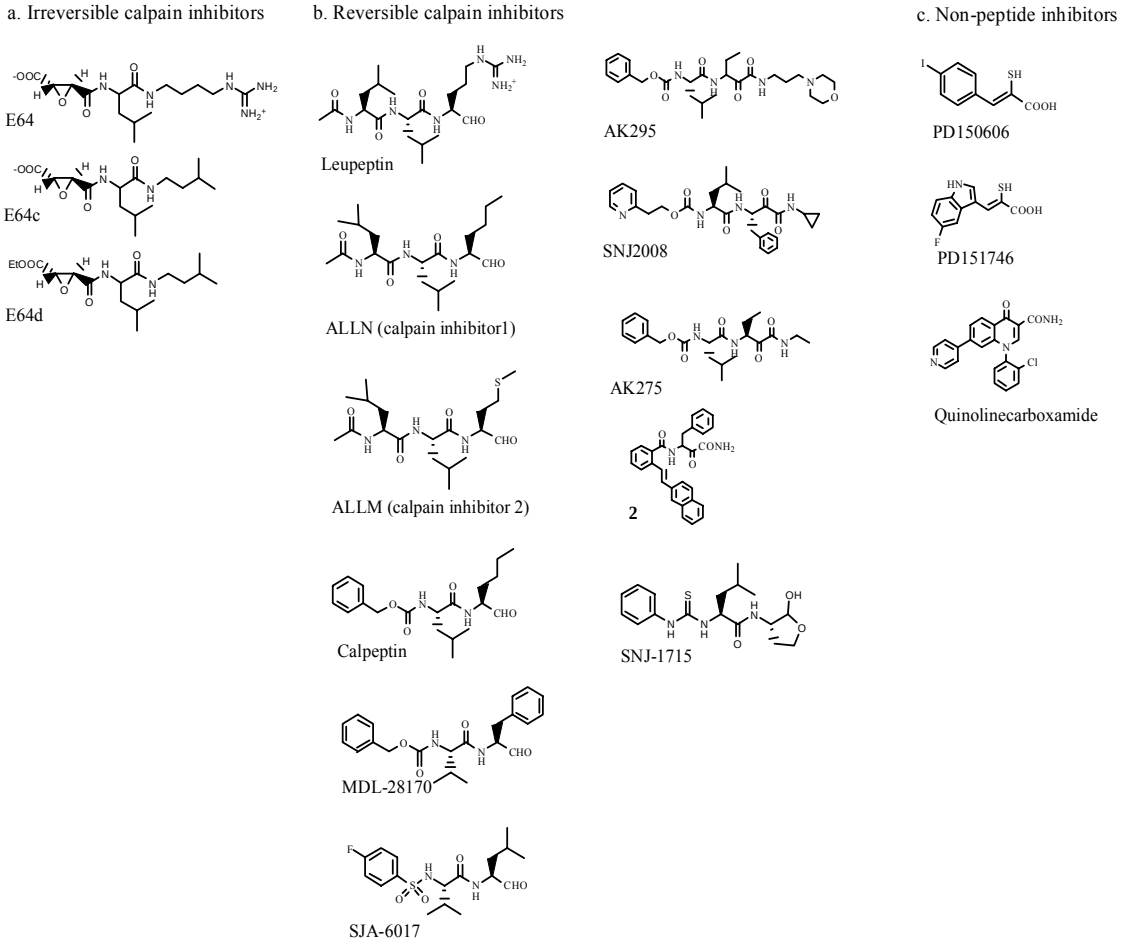


Figure 7. Typital calpain inhibitors.

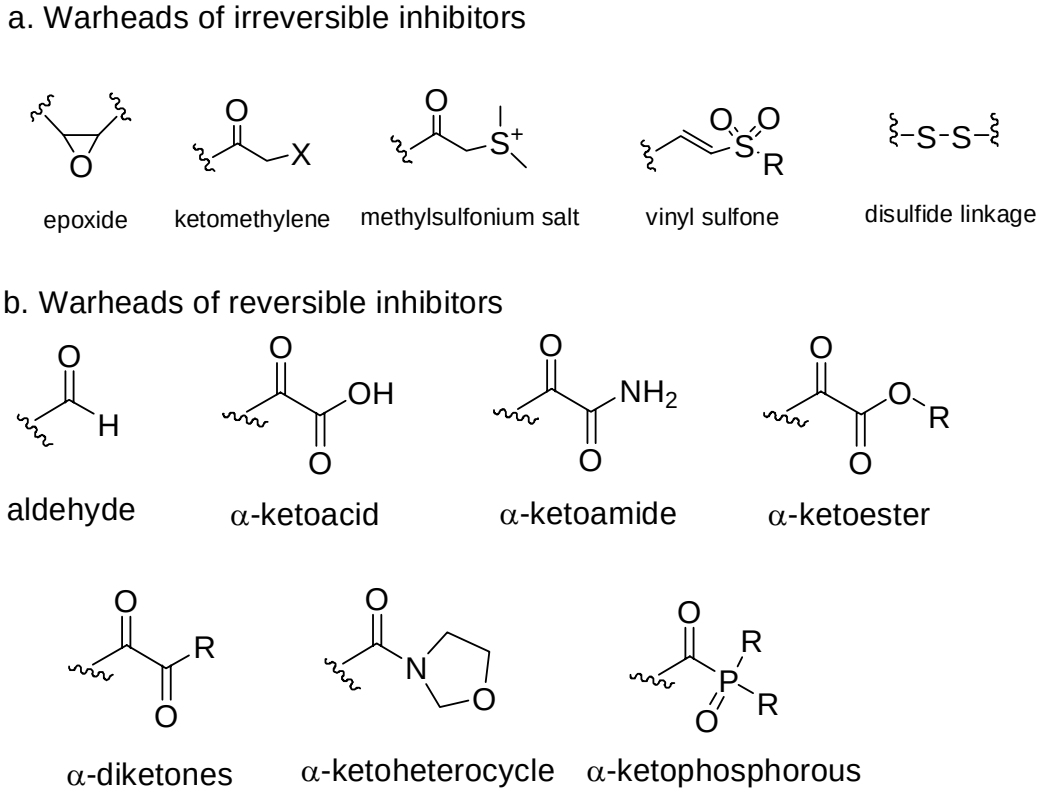


Figure 8. Functional groups of warheads in calpain inhibitors. R = substituted group; X = halogen.

Source: Donkor, I. O. A survey of calpain inhibitors. *Curr Med Chem* 2000, *7*, 1171-1188.[156] Modified with permission from Bentham Science Publishers Ltd.

Calpastatin is an endogenous calpain multiheaded[133] inhibitor that specifically inhibits both major isoforms of calpain, m- and μ-calpains.[13,134,135] It binds and inhibits calpain when calcium levels are high, and is released when calcium levels fall.[135] To date calpastatin is the only inhibitor that is completely specific for calpain.[135] Calpastatin does not inhibit cysteine proteases, such as papain, cathepsin B, bromelin, or ficin, and other proteases such as trypsin, chymotrypsin, plasmin, thrombin, pepsin, cathepsin D, or thermolysin.[17,136] Calpastatin is well conserved with greater than 70% identity across mammalian species. It is encoded by a single gene that has no obvious homologues.[135] The structure of calpastatin polypeptide (Figure 9) contains five domains, four homologous inhibitory domains of approximately 140 amino acids each and a unique N-terminal domain, termed domain L, with no inhibitory effect.[13,133,135] Each inhibitory domain contains three well-conserved subdomains, A, B and C.[133] Subdomains A and C of calpastatin interact at nM affinity with domain IV and domain VI of calpain, respectively.[135,137] While subdomain B interacts weakly with calpain in the absence of its flanking A and C regions[135] and is thought to bind to the catalytic site of domain II or domain III of calpain.[138] Each inhibitory domain is capable of inhibiting one calpain molecule,[133] so it is considered as calpain multiheaded inhibitor.

Plants are rich sources of calpain inhibitors. Peptidyl aldehyde calpain inhibitors, including leupeptin,[139] strepin P-1,[140] and staccopins P-1 and P-2,[141] can inactivate calpain by interacting reversibly with the active site thiol of the enzyme, but the problems are this inactivation is not selective to calpain and poor cellular permeability.[13] In addition, Hiwasa and colleagues[13,142] isolated damnacanthal, an anthraquinone compound, from the root of *Morinda critrifolia* can also effectively inhibit calpain. Some other calpain inhibitors isolated from plants were well summaried by Donkor.[13]

Although many potent calpain inhibitors have been isolated from natural sources, the problems of isolating compounds are selectivity over other cysteine proteases, cell permeability, and even metabolic stablility. So the modification of compounds was focused on improving these disadvantages.

Peptidyl aldehydes (Figure 7) are the largest group of calpain inhibitors. They can form a hemithioacetal intermediate by reacting with the active site thiol of the calpain catalytic cysteine. The prototypical compound[25] of this inhibitor class was leupeptin (Figure 7), which was isolated from streptomyces and can enhance neuronal survival in gerbils subjected to transient ischaemic injury[25,121] and reduce experimental spinal cord injury in vivo.[25,143] Leupeptin is a potent inhibitor of both μ-calpain and m-calpain, but also of plasmin, trypsin, papain, and cathepsin B.[13] The other problem is that it is not cell permeable because of the positively charged guanidinium group.

Modification of leupeptin produced a series of representative peptide aldehydes (Figure 7) which have greater cell permeability. Tripeptidyl aldehydes, ALLN (calpain

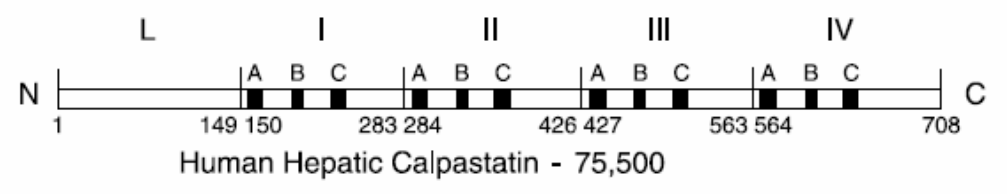


Figure 9. Domain structure of human hepatic calpastatin. N = amino terminal domain; C = carboxy terminal domain; I, II, III, IV and L = domain; A,B,C = subdomain.

Source: Wendt, A.; Thompson, V. F.; Goll, D. E. Interaction of calpastatin with calpain: a review. *Biol Chem* 2004, *385*, 465-472.[133] Adapted with the permission from Walter de Gruyter, Inc.

inhibitor 1) and ALLM (calpain inhibitor 2),[13,144] have been proven effective as calpain inhibitors. ALLN can reduce infarct size and neuronal damage in a rat model of focal ischaemia[25,116] and attenuate the systemic inflammatory response and multiple organ failure mediated by endotoxin in the rat.[25,145] ALLN and calpeptin have both been shown to reduce vessel restenosis following experimental transluminal angioplasty dilation of an atherosclerotic femoral artery in rabbits.[25,146] However, ALLN and ALLM are not specific for calpain. ALLN is a good inhibitor for cathepsin L (Ki = 0.5 nM) and ALLM inhibits cathepsin B (Ki = 100 nM).[13]

Calpeptin, MDL-28170 and SJA-6017 (Figure 7) are referred to be N-protected dipeptidyl aldehydes. Calpeptin is cell permeable and inhibits human platelet calpain 2 with an IC_{50} of 40 nM.[13] MDL-28170, which is also cell permeable,[8,13] can reduce neuronal damage in a rat model of cerebral ischemia[25,120] and reduce neuronal loss and improved locomotor functions in a mouse model of Parkinson's disease.[25,147] Besides, pre-injury administration of MDL-28170 in a rat model of diffuse brain injury can reduce neuronal injury. Post-injury administration of MDL-28170 by intraspinal microinjection in rats can effectively blocked elevation of calpain activity and attenuate axonal damage.[25] Post-injury administration of SJA-6017 improved neuronal functional outcome[12,25] in a mouse model of diffuse head injury. In addition, SJA-6017 showed neuroprotective efficacy via intravenous administration[9] in the rat retinal ischemia model. But its oral bioavailability was low due to the metabolic lability and low water-solubility.[9]

Peptidyl α-ketoamides (Figure 7) were found on development of selective calpain inhibitors with other peptidyl α-ketone compounds. The α-ketone compounds were supposed to reversibly form an enzyme-bound intermediate that results from the nucleophilic addition of the catalytic thiol of calpain to the electrophilic ketone of the inhibitor.[148] Because electrophilicity of α-ketone compounds is less than aldehyde, it is thought that α-ketone compounds may be more selective than aldehyde compounds. In general α-ketoamides were more effective toward calpain 1 and 2 than α-ketoesters,[14] so most recently published papers were focused on α-ketoamides

The early lead inhibitors, mostly aldehyde inhibitors, were hydrophobic and therefore poorly soluble in aqueous solutions.[148] α-ketoamides were developed to improve membrane permeability and the metabolic stability of aldehydes in vivo.[14,25] For example, AK275 (Figure 7) exhibits calpain inhibitory activity (Ki=109nM for porcine calpain I) and improved cell permeability.[148] AK295 (Figure 7), which has a morpholinopropyl group replacing the ethyl group of AK275, significantly improved solubility in aqueous solutions.[148,149] Both AK295[15] and AK275[150] provide significant neuroprotection against ischemic brain damage. Some other novel α-ketoamide derivatives that exhibit calpain inhibitory activity and can suppress neuronal damage in models of ischaemic and excitotoxic damage have been reported.[25,149,151,152]

In order to increase the water solubility, structure modification was going on P_3 position. SNJ-2008 (Figure 7), which has a pyridine group on the P_3 position, exhibited metabolic stability (about a 12-fold higher retinal AUC than SJA-6017), higher water-solubility, and would show retinal efficacy at a lower dose than aldehyde lead SJA-6017.[9]

An important inhibitor, compound **2** (Figure 7), was reported on 2002,[153] This naphthalene substituted compound has a *K*i of 6nM to human calpain[153] and represents one of the most potent calpain inhibitors, which are not derived from aldehydes. It also showed a moderate selectivity to cathepsin B (15-fold) and excellent selectivity to cathepsin L (1000-fold).[153] Compound **2** did not block non-cysteine proteases even up to higher mM concentrations.[153]

1.4.2 Allosteric Calpain Inhibitors

Researchers have recently begun to be interested in nonpeptide calpain inhibitors (Figure 7). The reason that non-peptide inhibitors are attractive is that they are not targeted against the active catalytic site but may interact with other allosteric sites of calpain that are important in catalytic activation.[25] This makes them selective calpain inhibitors over other cysteine proteases, even among the calpain subtypes.

PD150606 and PD151746 (*K*i=0.21 and 0.26μM for μ-calpain, respectively, Figure 7) are two α-mercaptoacrylic acid derivatives that are potent, selective and cell permeable inhibitors of calpain. X-ray crystal studies revealed that PD150606 inhibit calpain by binding to a hydrophobic pocket on domain VI of calpain small subunit.[154] PD151746 even showed approximately 20-fold selectivity for μ-calpain relative to m-calpain.[25,155] Carboximide derivatives such as quinolinecarboximides (*K*i=0.5μM, Figure 7) showed significant selectivity and exhibited almost 50-fold more inhibitory activity against μ-calpain relative to both cathepsin B and cathepsin L, members of cysteine proteases.[13,25] The α-mercaptoacrylic acids derivatives are cell-permeable and extremely selective for calpain (approximately 600-fold more selective for calpain relative to cathepsin B).[25] Some other nonpeptide compounds were well summaried by Donkor in 2000.[13]

1.4.3 SAR of Current Calpain Inhibitors

The binding mechanism for most of the nonpeptide calpain inhibitors has yet to be elucidated. They may interact with calpain at an allosteric site on the enzyme.[25] Calpain inhibitors targeted against the active catalytic site of calpain usually consist of an address region for enzyme recognition and an electrophilic group (warhead) that can react with the active site cysteine of the enzyme (Figure 6). In reversible inhibitors, the electrophilic group (usually aldehyde or α-keto carbonyl) form reaction

intermediates (hemithioacetal or hemithioketal) with the active site thiolate of the enzyme, which mimics the mechanism of hydrolysis reaction catalyzed by cysteine proteases .[156] All the requirements of SAR are summaried in Figure 10.

1.4.4.1 P_1 Position

The S_1 subsite of calpain can tolerate a wide variety of amino acids at the P_1 position of inhibitors.[13] The incorporation of aliphatic (Val, Nle, Leu, Abu, Cha, and Met) and aromatic (Phe, Tyr(O-Benzyl)) at the P_1 position resulted in compounds with good or moderate calpain I inhibitory activity.[157] Hydrophobic groups will enhance potency while incorporation of amino acids which are capable of hydrogen bonding does not increase potency (such as Ser, Thr, Gln and Tyr)[157] and is detrimental to binding.[13] Aromatic amino acids are less potent than aliphatic amino acids.[157] No substituted group, Gly, is detrimental to activity, compared with Ala.[157]

1.4.4.2 P_2 Position

It was generally thought that the P_2 substitute must be either L-valine or L-leucine in order to have potent calpain inhibitory activity.[13] P_2 prefer aliphatic side chain and amino acids at P_2 which are capable of hydrogen bonding does not increase potency.[157] Besides acidic or basic amino acids are not beneficial at P_2.[157] A recent study showed that peptides with D-amino acids at P_2 postion were also potent calpain inhibitors.[158,159] Donkor et al. replaced P_2 chiral carbon with nitrogen to make it urea-based peptidomimetic calpain inhibitors. The compounds mirrored the general SAR of peptidyl aldehyde calpain inhibitors and displayed greater selectivity for μ-calpain over cathepsin B.[3]

1.4.4.3 N-Terminal Capping

The N-terminal capping of the calpain inhibitors is one of the most interested regions in the development of selective and cellular permeability calpain inhibitors. It can tolerate bulky groups and provide an additional recognition element for binding to the S_3 and S_4 subsites of calpain. Polar pyridineethanol[9] group was used to increase potency and some of pyridineethanol compounds showed a good oral bioavailability. Groups used as N-terminal capping agents[13] are benzyloxycarbonyl, acetyl, aryl (or alkyl) sulfonyl, alkanoyl, substituted benzoyl, naphthoyl, thionaphthalene,[13,160] fluorine, xanthines[13,158] and pyridineethanol, Chromone,[7] 3,4-dihydro-1,2-benzothiazine 1,1-dioxide ring (Figure 11).[161,162]

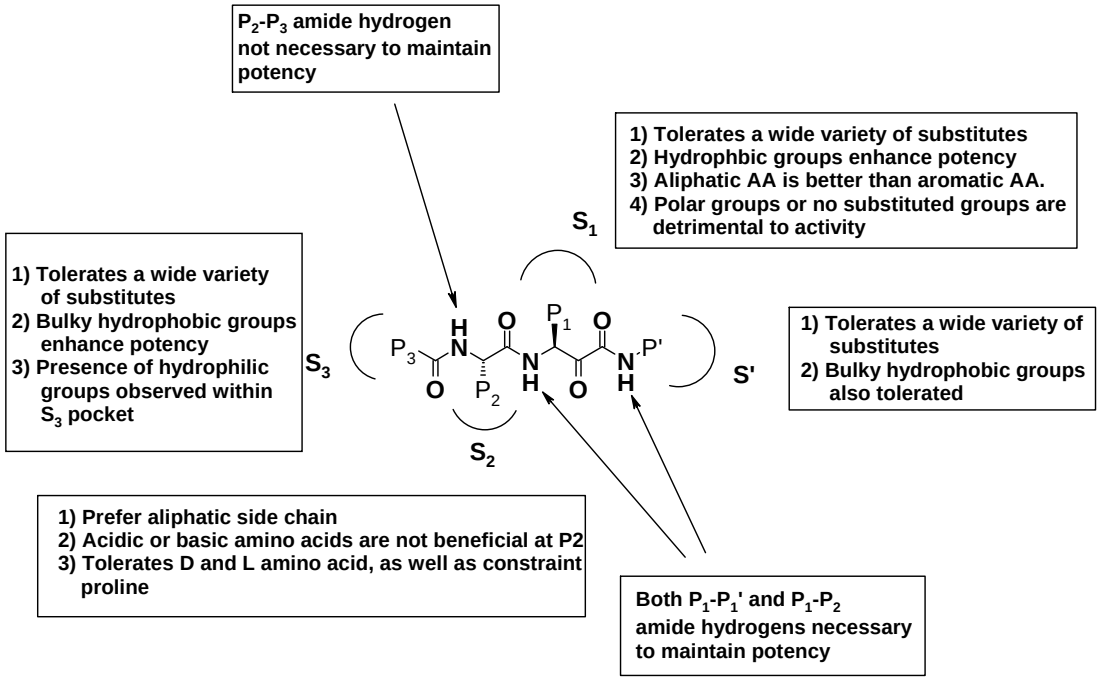


Figure 10. SAR of calpain inhibitors.

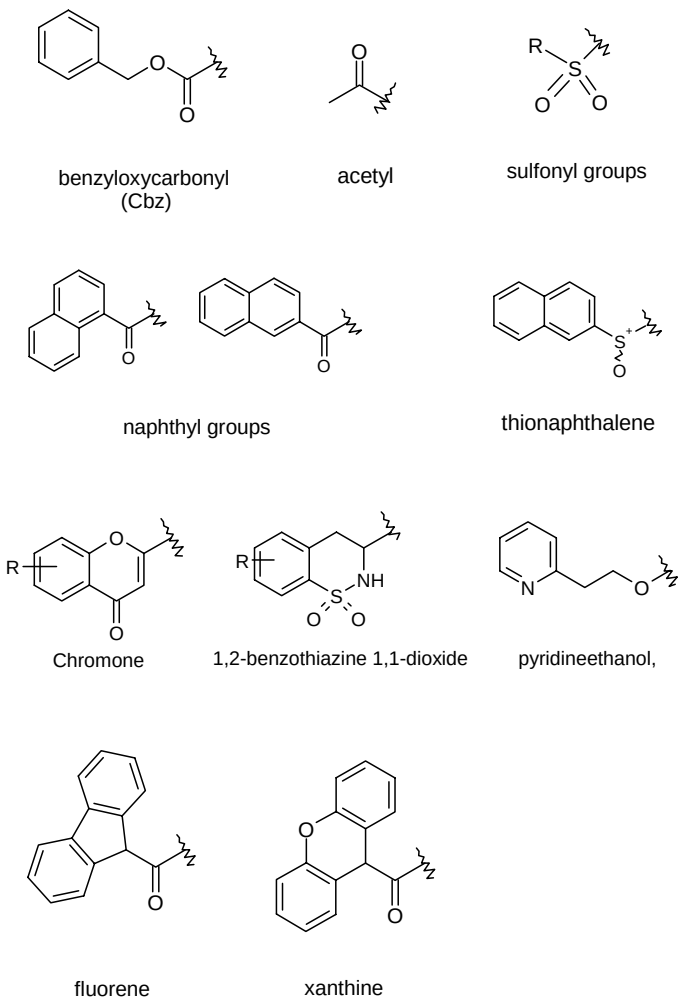


Figure 11. Amino terminal capping groups.

1.4.4.4 P' Position

Li and colleagues[14] demonstrated that the S_1' pocket can tolerate a wide variety of substitutes including large hydrophobic groups at the P_1' position. Polar morpholinopropyl group was also successfully introduced to increase the solubility of the compound without affecting activity.

1.4.4.5 Amide Hydrogen

Replacement of P_1-P_2 amide with ketomethylene isostere could decrease calpain inhibitory potency by 250 fold (Figure 12). This suggested that the hydrogen bonding between calpain and the P_1-P_2 amide hydrogen of the inhibitor is important for calpain inhibitory potency.[163] Donkor et al. showed that Gly271 is the active residue of calpain that makes the critical hydrogen bond between the calpain 1 and the P_1-P_2 amide hydrogen.[164] Donkor et al. also proved that P_1'-P_2' amide hydrogen is necessary to maintain potency, without it the activity of inhibitor could decrease by 380 fold (Figure 12).[164] Besides, Chaterjee et al.[160] and Donkor[3] proved that the P_2-P_3 amide hydrogen of a calpain inhibitor is not a strict requirement for calpain inhibition.

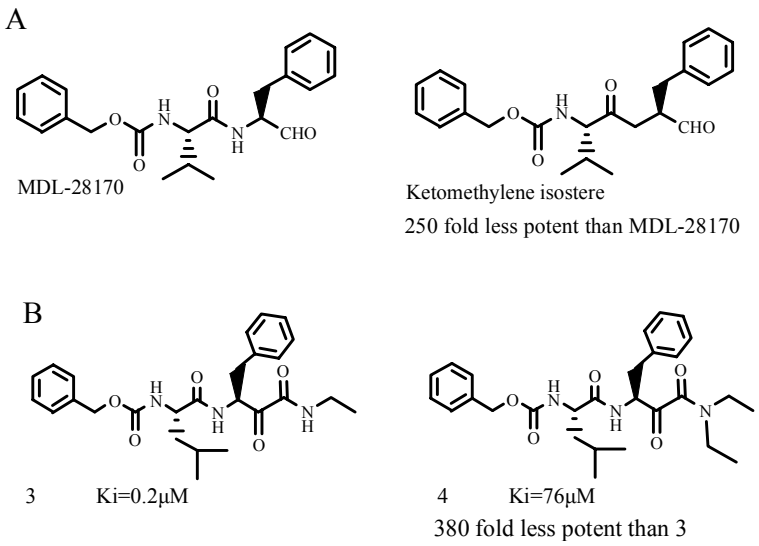


Figure 12. Importance of amide hydrogen.

CHAPTER 2. EXPLORATION OF DIAZOSULFONAMIDE DERIVATIVES AS CALPAIN INHIBITORS

2.1 Structural Requirements Study of Diazosulfonamide 1

2.1.1 Objective

Virtual screening of the NCI compound library led to the identification of diazosulfonamide **1** (Figure 1) as a new nonpeptide competitive inhibitor of calpain 1 (Donkor et al. Unpublished data). Analogs of diazosulfonamide **1 (**Figure 13) were synthesized to explore the structural requirements of **1** that are important for calpain inhibition as a prelude to the synthesis of novel sulfonamide-based peptidomimetic calpain inhibitors.

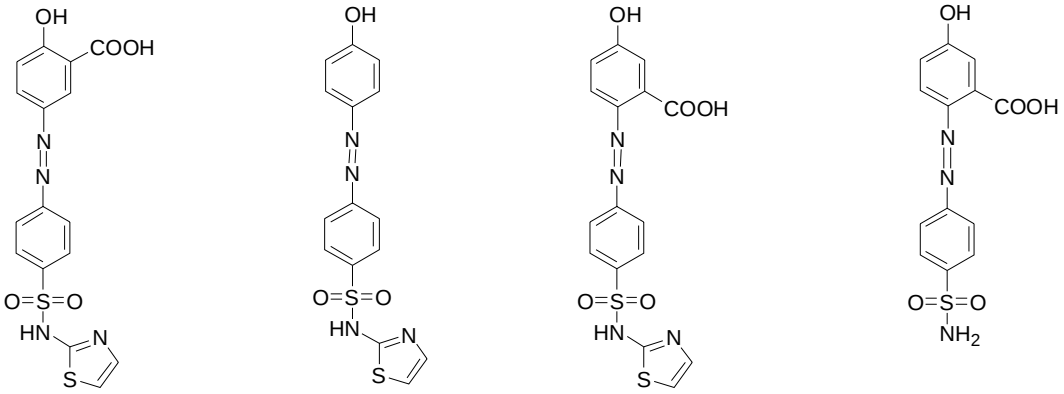
2.1.2 Chemistry

The synthesis of compounds **1** and **5-7** from the corresponding sulfanilamide (**8**) and sulfathiazole (**9**) is outlined in Scheme 1. The sulfonamides were diazotized using $NaNO_2$/HCl to obtain the corresponding diazonium salts, which were treated with salicylic acid or 3-hydroxybenzoic acid under basic condition (20% NaOH) to afford the target compounds after purification by flash chromatography.

2.1.3 Results and Discussion

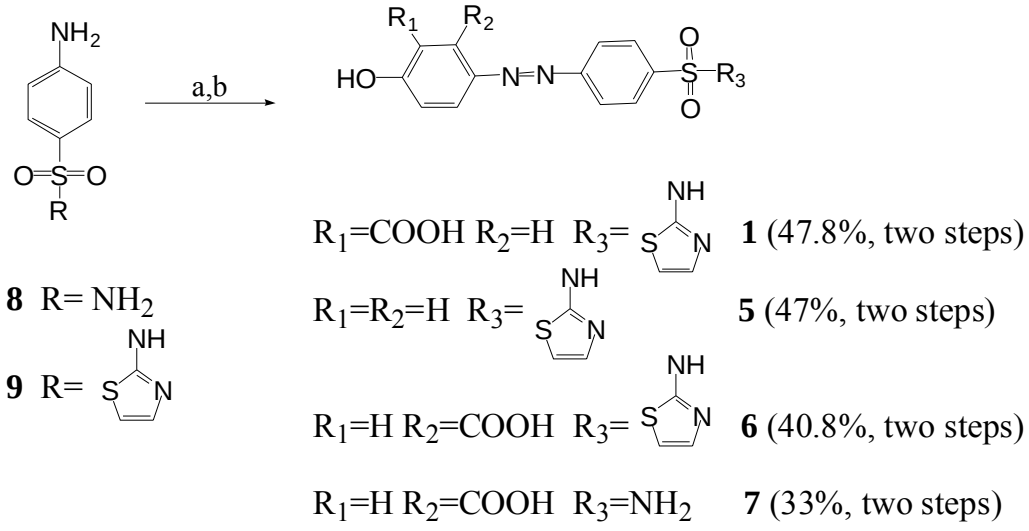
Diazosulfonamide **1** was identified by Donkor et al. (unpublished data) to be a nonpeptide competitive inhibitor of calpain 1. Figure 14 shows the interaction of the compound with active site residues of calpain. The carboxylic acid group of **1** formed a hydrogen bond with the NH of the imidazole side chain of His272 while the OH of Ser251 formed hydrogen bonds with the nitrogen of the thiazole ring and one of the sulfamoyl oxygen atoms of compound **1**. A fourth hydrogen bond was formed between the other sulfamoyl oxygen and Thr210.

Analogues of **1** (compounds **5-7** in Figure 13) were synthesized and studied as inhibitors of calpain with the goal of exploring the structural requirements of **1** that are important for inhibition of the enzyme as a prelude to the synthesis of novel sulfonamide-based peptidomimetic calpain inhibitors. The compounds were tested against porcine erythrocyte calpain 1 using Suc-Leu-Tyr-AMC as the substrate by following the procedure of Donkor et al.[165] The calpain inhibitory activity of the compounds is shown in Figure 13. Compound **5** was over 60-fold less potent than **1** suggesting that the hydrogen bonding interaction between the carboxylic acid group



1, K_i = 1.0±0.02 uM **5**, K_i = 63.2 uM **6**, K_i = 9.89±2.62uM **7**, K_i = 55.0±2.53 uM

Figure 13. Structure and calpain inhibitory activity of compound **1** and its analogues. *K*i = Inhibitory activity of the compounds versus porcine erythrocyte calpain 1.



Scheme 1. Synthesis of compounds **1** and **5-7**.
Reagents: (a) HCl, $NaNO_2$; (b) 20% NaOH.

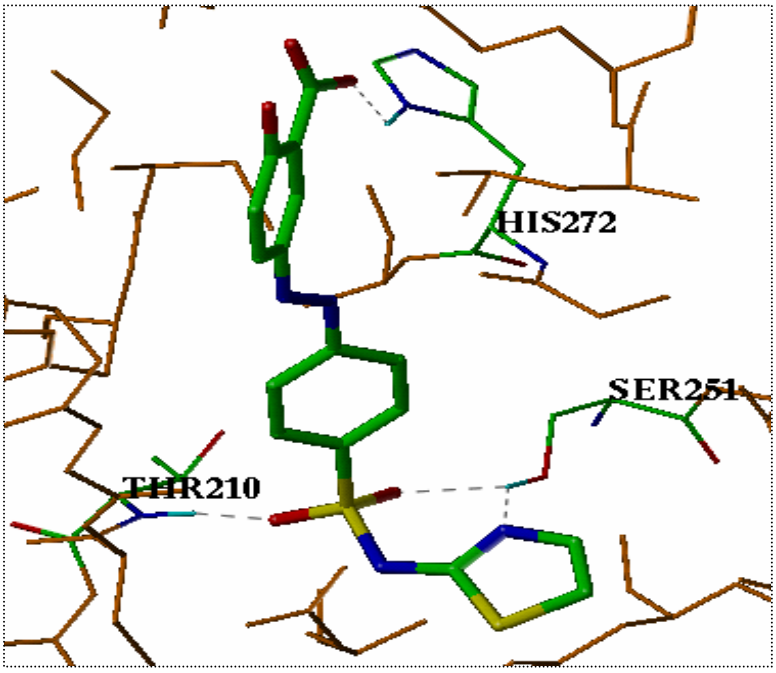


Figure 14. Hydrogen-bonding interactions between calpain 1 and compound **1**. Hydrogen atoms are removed for clarity, except those involved in hydrogen bonding. Hydrogen bonds are shown as black dashed lines. The atoms are colored as follows: all the protein residues are orange, except those involved in the hydrogen-bonding interactions; carbon = green; hydrogen = cyan; nitrogen = blue; oxygen = red; sulfur = yellow.

of **1** and His272 is important for calpain inhibition. Reposition of the carboxylic acid group meta to the phenolic hydroxyl as **6** led to about a 10-fold decrease in calpain inhibition, which further supports the significance of the hydrogen bonding interaction or salt bridge formation between the carboxylic acid and His272 to calpain inhibition. The thiazole ring, which formed three hydrogen bonds with active site residues, was also found to be very important for potent inhibition of calpain because removal of this group as in compound **7** resulted in about 55-fold and 6-fold decrease in calpain inhibition compared to compounds **1** and **6**, respectively.

2.1.4 Conclusion

Three analogues of compound **1** were synthesized and studied as inhibitors of porcine erythrocyte calpain 1. The compounds inhibited calpain 1 with K_i values ranging from 1 μM to 63.2 μM. The thiazole ring and the carboxylic acid substitutes of **1** were found to be very important for potent calpain inhibition.

2.2 Synthesis of Sulfonamide-Based Peptidomimetic Calpain Inhibitors

2.2.1 Objective

Most active site directed calpain inhibitors incorporate an electrophilic functionality for covalent interaction with the catalytic site thiolate of the enzyme.[13] This interaction was confirmed for the binding of leupeptin (PDB code 1TL9) and E64 (PDB code 1TL0) to engineered calpain 1.[125] Compound **1** does not possess an electrophilic center for covalent modification of calpain. Due to the significance of such an interaction for potent calpain inhibition, we hypothesized that peptidomimetic analogues of **1** that incorporate an electrophilic functionality for covalent interaction with the catalytic site thiolate residue should be potent inhibitors of calpain. Compounds **10**-**19** (Figure 15) were synthesized as novel sulfonamide-based peptidomimetic analogues of **1** to test this hypothesis. Within this series, compounds **18** and **19** were synthesized as ethylene bridged analgues of diazo derivatives **15** and **17**, respectively, since the diazo group is known to undergo reduction in vivo.

2.2.2 Chemistry

Compounds **10** and **11** were synthesized as shown in Scheme 2. Refluxing either 3- or 4-chlorosulfonyl benzoic acid **20** in $SOCl_2$ followed by treating the resulting chlorosulfonyl benzoyl chloride with L-phenylalanine methyl ester hydrochloride in the presence of DIPEA gave **21**, which was reacted with 2-amino-thiazole in the presence of pyridine to give sulfonamide **22** in 40% overall

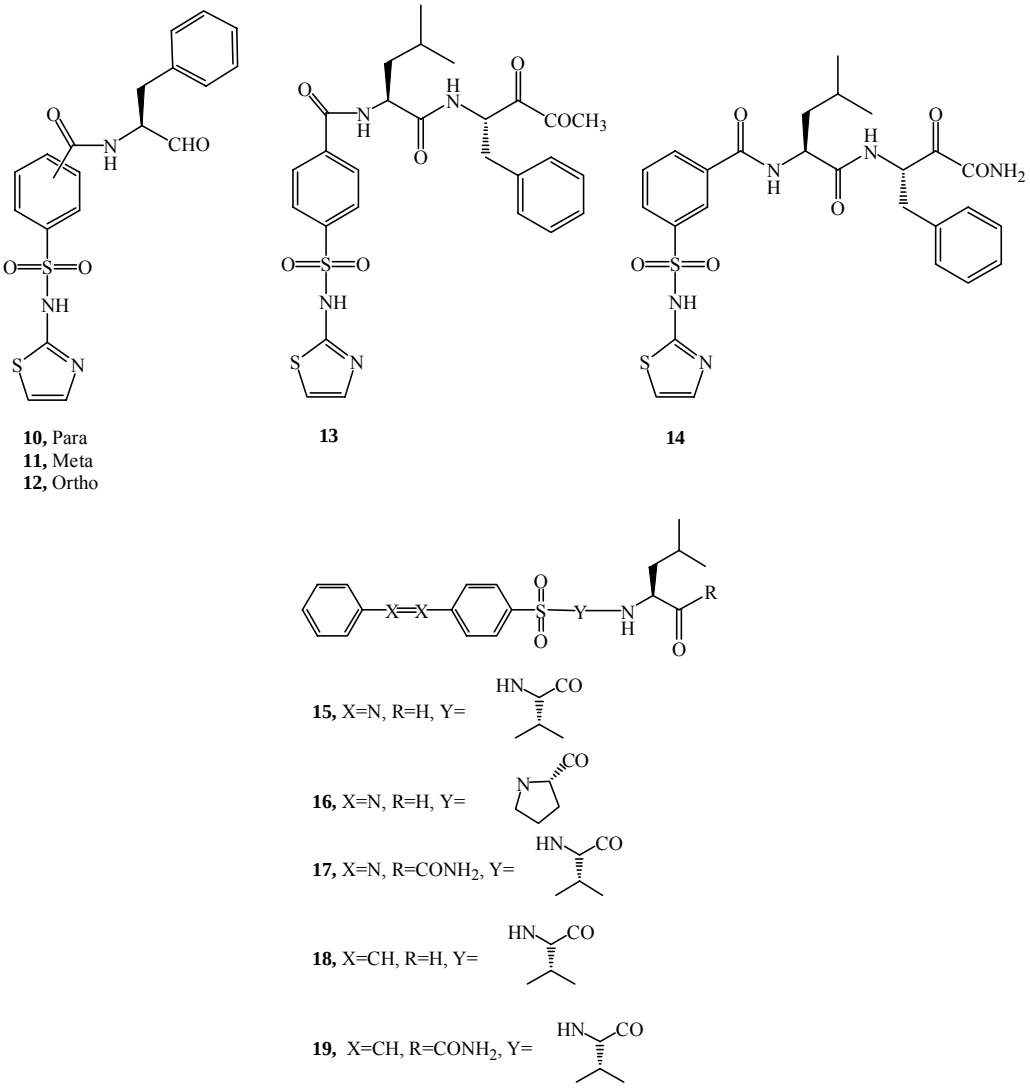
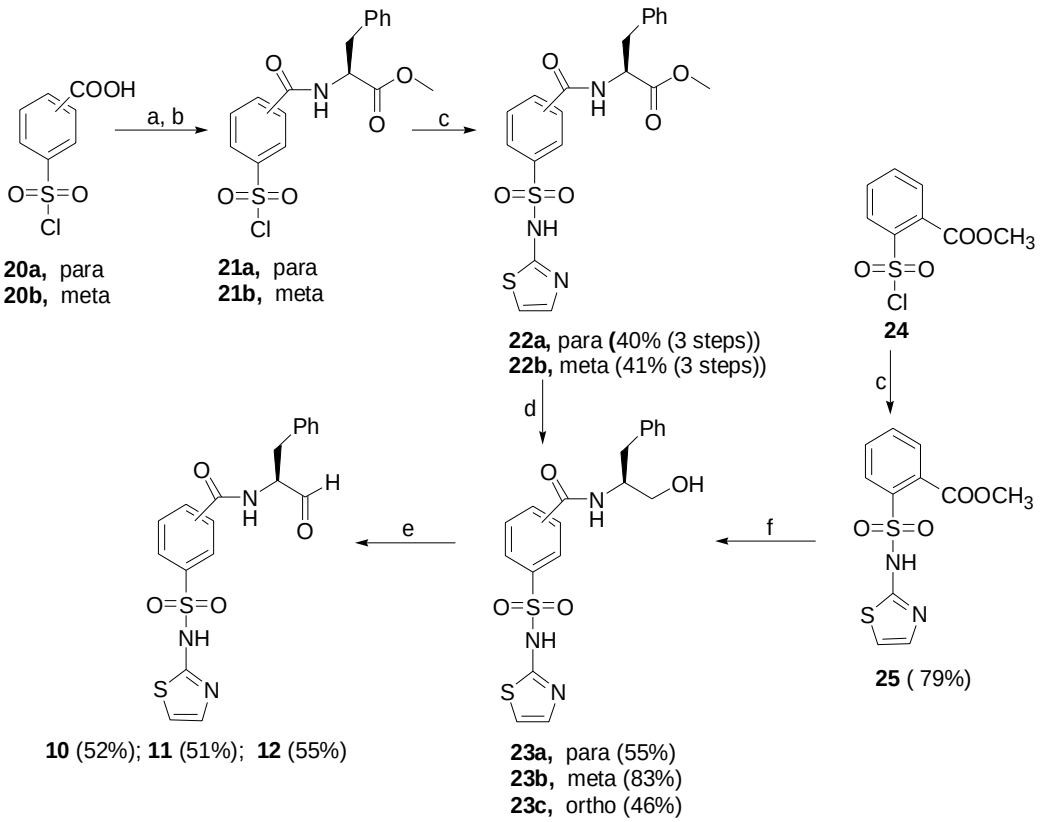


Figure 15. Sulfonamide-based peptidomimetic compounds **10-19.**



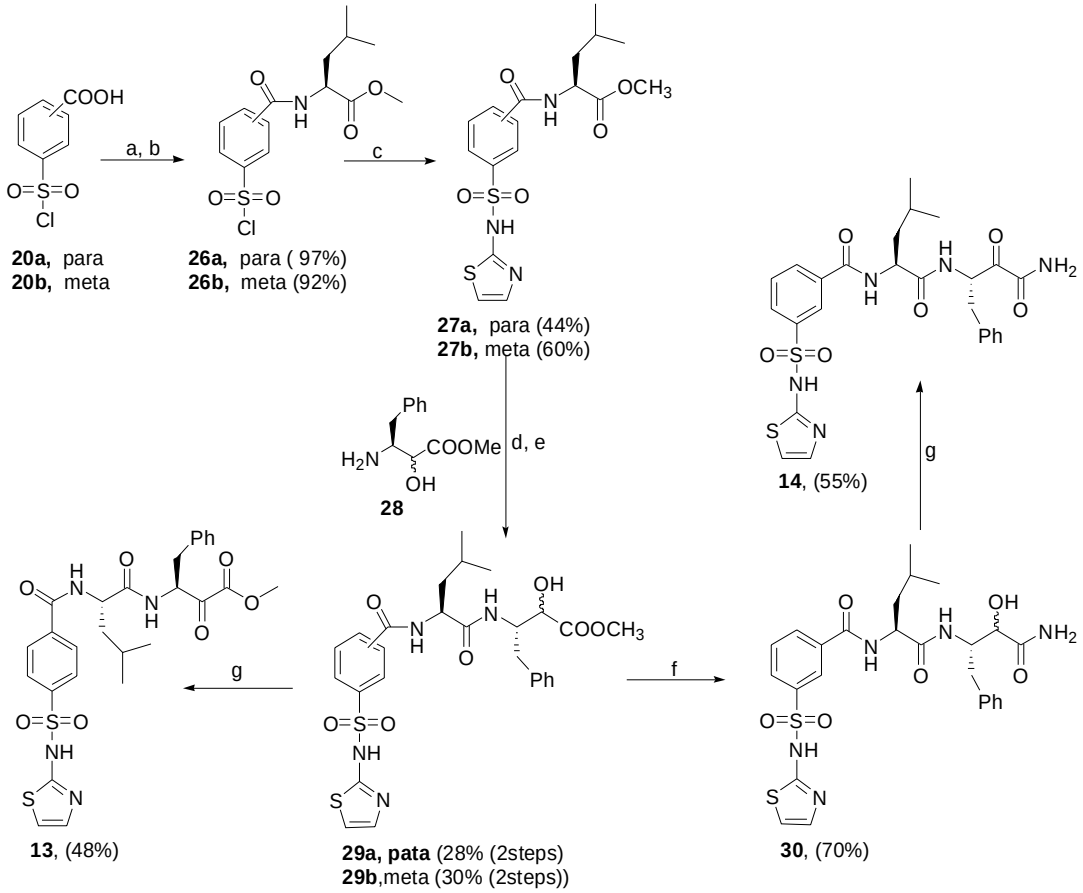
Scheme 2. Synthesis of compounds **10-12**.
Reagents: (a) $SOCl_2$, 1,2-Dichloroethane, Reflux, 1 h; (b) L-Phe-OMe.HCl, DIPEA, CH_2Cl_2, RT, 1 h; (c) 2-Amino-thiazole, Pyridine, RT, 16 h; (d) $LiBH_4$, 5 h; (e) $PySO_3$, DMSO, CH_2Cl_2, RT, 2 h; (f) DMF, 2-Amino-3-phenyl-propanol, 1,4-dioxane, microwave at 155ºC, 20 min.

yield. Reduction of the ester group of **22** with $LiBH_4$ afforded alcohol **23**, which was oxidized with $PySO_3$ in DMSO/DCM mixture to give target compounds **10** and **11**. Ortho substituted derivative **12** was synthesized by reacting methyl-(2-chlorosulfonyl)benzoate **24** with 2-amino-thiazole to give **25**. Microwave assisted coupling of **25** with 2-amino-3-phenylpropanol gave **23**, which was transformed to aldehyde **12** as described above.

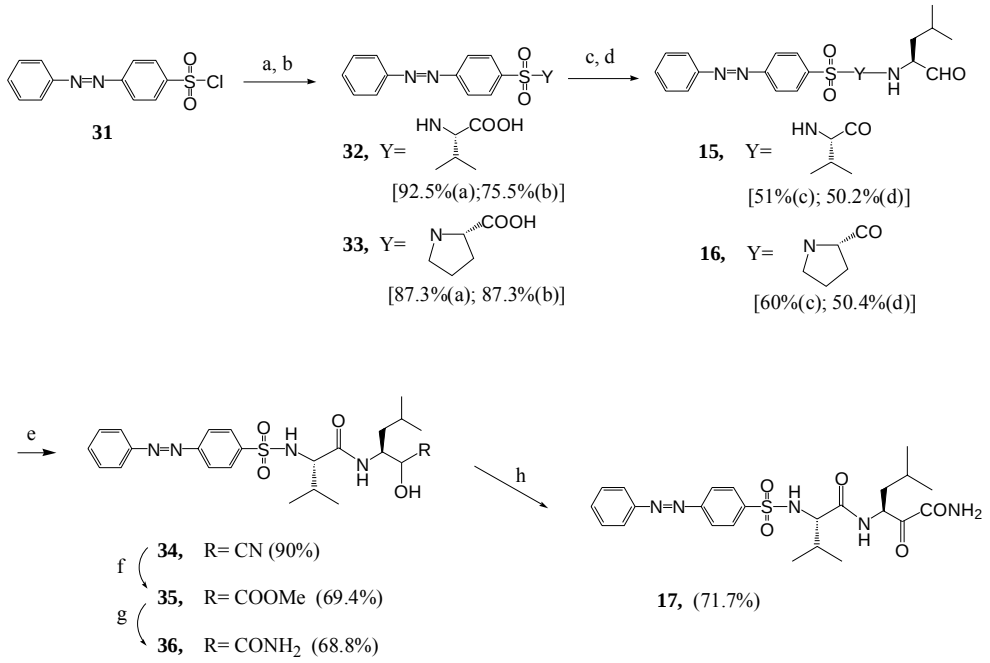
Dipeptide-sulfonamides **13** and **14** were synthesized as shown in Scheme 3. The appropriate chlorosulfonyl benzoic acid **20** was refluxed in $SOCl_2$, treated with L-valine methyl ester hydrochloride in the presence of DIPEA followed by reaction with 2-amino-thiazole and ester hydrolysis with 1.0 N NaOH to give sulfonamide **27**. This was coupled with **28**, which was synthesized as previously reported by Donkor et al[166] to give beta-hydroxy ester derivative **29**. Oxidation of **29** with $PySO_3$/DMSO mixture afforded target compound **13**. Attempted hydrolysis of the ester functionality of **13** to obtain the α-ketoacid derivative was unsuccessful. Treatment of **29** with $MeOH/NH_3$ gave α-hydroxy-β-animo amide derivative **30**, which was oxidized with $PySO_3$/DMSO mixture to give **14**.

The synthesis of compounds **15-17** is outlined in Scheme 4. Compounds **15** and **16** were synthesized starting with the commercially available 4-phenylazobenzene sulfonyl chloride **31**, which was treated with either L-valine methyl ester hydrochloride or L-proline methyl ester hydrochloride in the presence of DIPEA to give **32** and **33**, respectively. Basic hydrolysis of the ester groups of these compounds followed by couplying with L-leucinol and oxidation with Dess-Martin reagent gave target compounds **15** and **16**. The aldehyde **15** was reacted with KCN to give cyanohydrin **34** and hydrolysis of the cyano group gave α-hydroxy-β-animo acid ester **35**. Treatment of **35** with $MeOH/NH_3$ gave α-hydroxy-β-animo amide **36**, which was oxidized with $PySO_3$/DMSO mixture to give **17**.

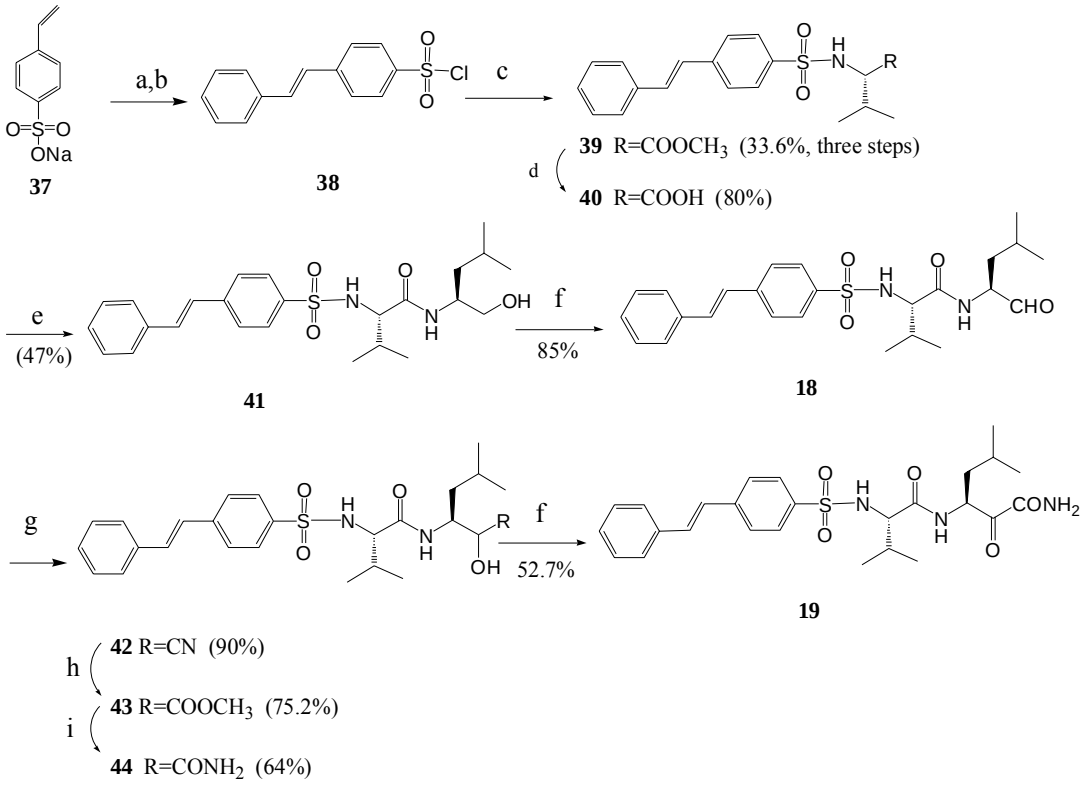
The synthesis of compounds **18** and **19** is outlined in Scheme 5. The compounds were synthesized as described for the synthesis of diazo compounds **15-17**. 4-Styrene sulfonic acid sodium salt **37** was converted to 4-styryl-phenylsulfonic acid sodium salt using Heck reaction, which is known to generate products with *E* configuration. We confirmed the geometry of the compounds using 2D C-H correlation spectroscopy. Refluxing of the phenylsulfonic acid sodium salt in $SOCl_2$ gave sulfonyl chloride **38**, which was treated with L-valine methyl ester hydrochloride in the presence of DIPEA to give sulfonamide **39**. Hydrolysis of the ester group of **39** followed by coupling of the resultant acid with L-leucinol afforded **41**, which was transformed to aldehyde **18** and alpha-ketoamide **19** as described for the synthesis of the corresponding diazo derivatives **15** and **17** in Scheme 4.



Scheme 3. Synthesis of dipeptide-sulfonamides **13** and **14**.
Reagents: (a) $SOCl_2$, 1,2-Dichloroethane, Reflux, 1 h; (b) L-Val-OMe.HCl, DIPEA, CH_2Cl_2, RT, 1 h; (c) 2-Amino-thiazole, Pyridine, RT, 16 h; (d) 1.0 N NaOH; (e) HBTU, DIPEA, DMF; (f) 7.0 N NH_3 in MeOH, 16 h; (g) $PySO_3$, DMSO, CH_2Cl_2, RT, 2 h.



Scheme 4. Synthesis of compounds **15-17**.
Reagents: (a) Pyridine, L-Val-OMe.HCl or L-Pro-OMe.HCl, RT, 20 h; (b) 1.0N NaOH, MeOH, 55ºC, 2 h; (c) L-leucinol, CDI, THF, CH_2Cl_2, RT, 96 h; (d) Dess-Martin reagent, CH_2Cl_2; (e) $NaHSO_3$/KCN; (f) con. HCl/MeOH; (g) 7.0N NH_3 in MeOH, 16 h; (g) $PySO_3$, DMSO.



Scheme 5. Synthesis of compounds **18-19**.
Reagents: (a) bromobenzene, $Pd(OAc)_2$, TEA, $(o\text{-}Tol)_3P$; (b) $SOCl_2$, DMF; (c) l-Val-OMe hydrogenchloride, DIPEA, pyridine; (d) 4.0N NaOH, MeOH; (e) l-leucinol, TEA, Mukayaima's regaent, DMF; (f) $PySO_3$, DMSO; (g) $NaHSO_3$/KCN; (h) con. HCl/MeOH; (i) 7.0N NH_3 in MeOH.

2.2.3 Results and Discussion

The sulfonamide-based peptidomimetic compounds **10**-**19** were synthesized and tested as inhibitors of porcine erythrocyte calpain 1 to test the hypothesis that derivatives of diazosulfonamide **1** that incorporate an electrophilic group for covalent interaction with Cys115 at the active site of calpain would be potent inhibitors of the enzyme. Table 2 shows the results of this study. The compounds inhibited calpain 1 with K_i values ranging from 0.009 μM to 29.8 μM. Structure requirement study of compound **1** showed that the thiazole ring is very important for potent calpain inhibition. We therefore synthesized compounds **10**-**14** to determine if appending an electrophilic functionality to the phenylsulfathiazole substructure of **1** would result in potent calpain inhibition. Compounds **10**-**12** were designed as pseudo dipeptide derivatives while **13** and **14** were designed as pseudo-tripeptide derivatives. Generally, the pseudo-tripeptides were more potent calpain inhibitors than the pseudo-dipeptide derivatives. Compound **14** with K_i of 62 nM was the most potent member of the series. The position of the peptidyl substitute influenced calpain inhibition with the meta position (as in **11** and **14**) being the most preferred position and the ortho position (as in **12**) being the least preferred position.

Calpain inhibitors such as MDL28170 were discovered as a result of N-terminal capping of dipeptide aldehyde inhibitors of calpain.[13] The N-terminal capping group occupies the S_3/S_4 subsites of the enzyme. Compounds **15**-**17** were synthesized to investigate if the *N*,*N'*-diphenyl sulfonyl group of compound **1** could serve an effective N-terminal capping functionality to enhance calpain inhibition. The compounds were potent inhibitors of calpain 1 and displayed K_i values between 16 nM and 273 nM. Compound **15** with L-valine as the P_2 substitutent was more potent than **16** with a P_2 proline substitutent, which is consistent with previous reports that the S_2 subsite of calpain prefers small hydrophobic groups at the P_2 position of inhibitors.[13] The alpha-ketoamides **17** and **19** were as potent as the corresponding aldehydes **15** and **18**, respectively. The diazo bridge of **1** was not involved in hydrogen bonding interactions with active site residues (Figure 14). Therefore, we hypothesized that replacement of the diazo bridge with an ethylene linker should not be detrimental to calpain inhibition. This replacement was inspred by the fact that the diazo group of diazosulfonamides (e.g., protosil) is known to undergo reduction in vivo. Compounds **18** and **19** were then synthesized as deaza analogues of **15** and **17**, respectively, and found to be potent inhibitors of calpain with **19** (K_i = 9 nM) being the most potent member of all the novel sulfonamide-based peptidomimetic inhibitors derived from diazosulfonamide **1**. Indeed, compound **19** is one of the most potent calpain inhibitors known to date. This compound is especially interesting because it is an alpha-ketoamide derivative and so unlike aldehyde calpain inhibitors should not undergo oxidation of the critical electrophilic functionality in vivo. Thus, **19** should have longer in vivo exposure compared to its aldehyde congener **18** however this awaits future pharmacokinetic investigation.

Table 2. Inhibition of Porcine Erythrocyte Calpain 1 by Compounds **10**-**19**.

Compound	[a]K_i (μM)
10	4.36
11	0.5
12	29.8
13	8.42
14	0.062
15	0.018
16	0.273
17	0.016
18	0.014
19	0.009

[a]K_i values were determined by plotting 1/ν versus I to give intersecting lines with correlation coefficient ≥ 0.95.

2.2.4 Conclusion

Sulfonamide-based peptidomimetic compounds **10**-**19** have been synthesized as potent calpain 1 inhibitors. The compounds are hybrid molecules of substructures derived from diazosulfonamide **1** and dipeptide aldehyde and dipeptide alpha-ketoamides. Introduction of the electrophilic functionality (i.e., the aldehyde and alpha-ketoamide) groups significantly enhanced calpain inhibition. Sulfonamide-based peptidomimetic analogue **19** with K_i of 9 nM was the most potent member of the series. It is twice as potent as MDL28170 (K_i = 20 nM) and over 100-fold more potent than the lead diazosulfonamide **1**.

CHAPTER 3. DETERMINATION OF THE ANTI-TUMOR ACTIVITY OF THE SULFONAMIDE-BASED PEPTIDOMIMETIC CALPAIN INHIBITORS

3.1 Objective

Cancer is thought to be deficiency or upregulated function/expression of certain functional proteins, which are substrates of various proteolytic enzymes. Hence, it is possible to influence a cascade of signal transduction events by regulating the activity of a single proteolytic enzyme. As discussed in the introduction (Section 1.3.4), calpain is one of these proteolytic enzymes and has been implicated a molecular target for cancer therapy. Currently, 75% of cancer deaths are due to epithelial cancers. The five year survival rates for patients with disseminated melanoma is less than 5% with an average survival time of 6-10 months.[167] Common anticancer drugs, such as taxol, have shown lack of clinical efficacy. Inhibition of calpain suppresses cell growth and promotes apoptosis in a variety of human cancer cell lines.[168-171] Thus, calpain could offer a novel target for the discovery of new anticancer agents.

Although *K*i values as determined in cell free system evaluate the intrinsic inhibitory potency of compounds, drug effects in whole cell systems are potentially confounded by factors including drug stability, cellular penetration and/or non-specific binding. To explore the anti-proliferative activities of calpain inhibitors in whole cell systems, a selected set of novel sulfonamide analogs and peptidomimetic inhibitors were evaluated in human melanoma cell A375 and mouse melanoma cell B16F1. Evaluation of the compounds against non-cancer cell line is going on.

3.2 Results and Discussion

Compounds **1, 6, 11, 12** and **15-19** were tested against A375 human melanoma cells and mouse B16F1 melanoma cells to explore the anti-proliferative activities of calpain inhibitors. Studies were performed according to the protocol described in Section 4.3 and the results are summaried in Table 3. Compounds **1, 6, 11** and **12** did not show cell growth inhibition at 20 μM. This could be due to poor cell penetration and/or weak calpain inhibition. The ClogP values of the compounds ranges from 2 to about 3.7.

Compounds **15-19** showed antiproliferation activity at 20 μM and were selected for determination of the concentration required to inhibit melanoma cell growth by 50% (i.e., GI_{50}). The compounds displayed moderate antiproliferation activity with GI_{50} values ranging from 4 μM to 22 μM. Compound **16** (Figure 15) with proline as the P_2

Table 3. Structures and Activity of Calpain Inhibitors against Melanoma Cells (A375 and B16F1) and Porcine Erythrocyte Calpain 1.

Compound	A375	B16F1	$^{d}K_i$ (μM)	^{e}cLogP
	$^{b}GI_{50}$± ^{c}s.e. (μM)	GI_{50}± s.e. (μM)		
Taxol	0.009±0.001	0.021±0.0009		
1	a	ND	1.0	3.7
6	a	ND	9.89	3.1
11	a	ND	0.5	2.1
12	a	ND	29.8	2.1
15	12.9±1.5	13.7±5.0	0.018	5.5
16	4.1±0.5	13.7±1.7	0.273	4.6
17	22.3±2.1	18.7±3.0	0.016	4.0
18	9.4±2.1	10.3±1.9	0.014	5.7
19	16.0±1.3	17.2±2.8	0.009	4.2

a = cell growth inhibition <2% at 20μM.
$^{b}GI_{50}$ = half maximal growth inhibition
^{c}s.e.= standard error
$^{d}K_i$ values were determined by Dixon plots using the average of assays and plotting 1/v versus I to give intersecting lines with correlation coefficient ≥0.95.
^{e}cLog P is the calculated partition coefficients of the inhibitors and were determined with ChemDraw Ultra ver. 9.0.

substitutent was the most effective member of the series for inhibiting the growth of the human melanoma cell line A375. Compound **16** with K_i of 273 nM was over 3-fold more potent than **15** with K_i of 18 nM as inhibitor of A375 cell growth. Thus, there appears to be no correlation between calpain inhibition and antiproliferation activity of the compounds. However, the compounds were equipotent versus the mouse B16F1 cell line. Generally, there was limited variability in the GI_{50} values of the compounds versus the mouse cell line (GI_{50} 10.3 μM to18.7 μM) compared to the human cell line (GI_{50} 4.0 μM to 22.3 μM). Compounds **18** and **19** are deaza analogues of **15** and **17**, respectively. The were more effective against the human A375 cells compared to **15** and **17** but were equipotent with these compounds versus the mouse melanoma cell line. Compounds **17** and **19** are α-ketoamide analogues of aldehydes **15** and **18**, respectively. Generally, the aldehydes were better antiproliferative agents compared to the corresponding α-ketoamides presumably due to their greater lipophilicity, which should facilitate cell penetration. Nonetheless, α-ketoamide **17** and **19** may be more interesting than aldehydes **15** and **18** due to the superior cellular stability of the α-ketoamide warhead.

3.3 Conclusion

In this study, we report that the aldehyde and α-ketoamide calpain inhibitors showed moderate GI_{50} values (4 μM to 22 μM) for the growth inhibition of both human melanoma cell line A375 and mouse melanoma cell line B16F1. Compound **16** was the most effective antiproliferative agent (GI_{50} 4 μM) of the series. Compounds **18** and **19** with an ethylene bridge were better inhibitors of calpain and were also better antiproliferative agents than those with the diazo bridge (**15** and **17**). Besides, the ethylene brideg unlike diazo brideg will not undergo reduction in vivo. These characters make styrenephenyl group to be excellent group for further studies.

CHAPTER 4. EXPERIMENTAL SECTION

4.1 General

All reagents and solvents were purchased from Sigma Aldrich, Fischer Scientific, TCI, and Calbiochem, and were used without any further purification. Thin layer chromatography (TLC) was performed on silica gel chromatogram plates purchased from Analtech, Inc. Fisher silica gel S732-25 (100-400 mesh) was used for column chromatography. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Molecular masses were determined with electron spray ionization mass spectra (ESI-MS) in Bruker/Hewlett Packard Esquire LC/MS instrument. Nuclear magnetic resonance (NMR) spectra for ^{1}H NMR and ^{13}C NMR were recorded on Bruker ARX 300 instrument and Varian Inova-500 MHz. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. The chemical shifts (δ) are reported in parts per million (ppm) relative to TMS, and coupling constants (J) were reported in hertz. Splitting patterns were indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. IR spectra (neat) were recorded on a Perkin Elmer precisely Spectrum 100 FT-IR spectrophotometer, and the representative absorption bands were reported. Microwave reactions were performed on CEM Discover Benchmate. Elemental analyses (C, H, N) were performed by Atlantic Microlab Inc., Norcross, GA and are within ± 0.4% of the theoretical values.

4.2. Chemistry

4.2.1 General Procedure

General procedure 1. Synthesis of diazosulfonamides. The appropriate aromatic amine (6 mmol) was dissolved in a mixture of concentrated HCl (3.4 mL) and H_2O (14 mL) and diazotized by the dropwise addition of a solution of 17% $NaNO_2$ (7.2 mmol) at 0 °C and the mixture was stirred 40 min. Phenol, salicylic acid or 3-hydroxy benzoic acid (6 mmol) was dissolved in 20% NaOH (12 mL), cooled to 0 °C, and a solution of the appropriate aryldiazonium salt was slowly added. The resultant colored mixture was stirred for 1 h at 0 °C and the product was precipitated by addition of 20% HCl. It was recovered and recrystallized from acetone/water mixture.

General procedure 2. Ester reduction. $LiBH_4$ (40.4 mmol) was carefully added carefully to a solution of appropriate ester (21.6 mmol) in anhydrous THF (75 mL) at -15 °C for 5 min followed by stirring at RT for 0.5-2 h (checked by TLC). Excess $LiBH_4$ was destroyed by the dropwise addition of acetone at 0 °C followed by extraction with CH_2Cl_2 and purification of the product by flash chromatography.

General procedure 3. Dess-Martin oxidation. Dess-Martin reagent (17.2 mmol) was slowly added to an ice cooled solution of the appropriate alcohol (15.6 mmol) in anhydrous CH_2Cl_2 (80 mL). After 10 min, the ice bath was removed and the milky reaction mixture was stirred at RT for 2-4 h (checked by TLC). A solution of $Na_2S_2O_3$ (160 mmol) in saturated $NaHCO_3$ was added and the mixture was stirred for additional 10 min at RT. After separation, the aqueous layer was extracted with CH_2Cl_2 (3 x 30 mL) and the combined organic layer was washed successively with $NaHCO_3$ solution, water, and brine followed by drying ($MgSO_4$), concentration, and purification either by flash chromatography or crystallization.

General procedure 4. Pyridine-sulfur trioxide oxidation. A solution of $PySO_3$ complex (7.18 mmol) in DMSO (15 mL) was added dropwise to an ice-cooled solution of the alcohol (0.798 mmol) and DIPEA (7.36 mmol) in CH_2Cl_2/DMSO (30 mL) and the mixture was stirred for 1 h at 0 °C. The reaction mixture was diluted with EtOAc (300 mL), washed with aqueous 1N HCl (2 x 30 mL), saturated aqueous $NaHCO_3$ (2 x 20 mL), and brine. The organic phase was recovered and dried over $MgSO_4$ followed by concentrated and purification either by flash chromatography or crystallization.

4.2.2 Synthesis of Compounds 1, 5, 6, and 7

4-((4-Hydroxyphenyl)diazenyl)-*N*-(thiazol-2-yl)benzenesulfonamide (5). Compound **5** was synthesized as described in general procedure 1 as brown solid in 47% yield. m.p.252-253 ºC. ^{1}H NMR (DMSO-d_6) δ 12.8 (s, 1H), 10.44 (s, 1H), 7.87-7.95 (m, 4H), 7.81 (d, J=9.0, 2H), 7.26 (d, J=4.5, 1H), 6.94 (d, J=9.0, 2H), 6.84 (d, J=4.5, 1H). MS (ESI) 458.9 $[M-H]^-$. IR (cm^{-1}) 3355 (OH). Anal. Calcd. for: $C_{15}H_{12}N_4O_3S_2$, C, 49.99; H, 3.36; N, 15.55; S, 17.79. Found: C, 49.81; H, 3.37; N, 15.47; S, 17.75.

2-Hydroxy-5-((4-(*N*-thiazol-2-ylsulfamoyl)phenyl)diazenyl)benzoic acid (1) Compound **1** was obtained as brown solid in 47.8% yield using the general procedure 1. m.p.252-254 ºC. ^{1}H NMR (DMSO-d_6) δ 12.87 (s, 1H), 8.36 (d, J=2.7, 1H), 8.11 (d, J=2.4, 1H), 8.08 (d, J=2.7, 1H), 7.98 (s, 3H), 7.29 (d, J= 4.5, 1H), 7.17 (d, J=4.5, 1H), 6.87 (d, J=4.8, 1H). MS (ESI) 402.9 $[M-H]^-$. IR (cm^{-1}) 3013 and 3095 (br, OH), 1659 (C=O). Anal. Calcd. for: $C_{16}H_{12}N_4O_5S_2$•0.375H_2O, C, 46.74; H, 3.13; N, 13.63; S, 15.60. Found: C, 46.75; H, 3.15; N, 13.61; S, 15.59.

5-Hydroxy-2-((4-(*N*-thiazol-2-ylsulfamoyl)phenyl)diazenyl)benzoic acid (6). Compound **6** was obtained as brown solid in 40.8% yield as described in general procedure 1. m.p.213-214ºC. ^{1}H NMR (DMSO-D6) δ 12.98 (br, 2H), 10.66 (s, 1H), 7.95 (d, J=8.7, 2H), 7.84 (d, J=8.7, 2H), 7.65 (d, J=8.7, 1H), 7.25 (d, J=4.8, 1H), 6.97-7.04 (m, 2H), 6.84 (d, J=4.8, 1H). MS (ESI) 402.9 $[M-H]^-$. IR (cm^{-1}) 3339 (OH),

1713 (C=O). Anal. Calcd. for: $C_{16}H_{12}N_4O_5S_2$•0.25 EtOAc. C, 47.88; H, 3.31; N, 13.14; S, 15.04. Found: C, 47.59; H, 3.40; N, 13.06; S, 14.87.

5-Hydroxy-2-((4-sulfamoylphenyl)diazenyl)benzoic acid (7) Compound **7** was obtained as brown solid in 35.5% yield by following general procedure 1. m.p. 245-247ºC. ^{1}H NMR (DMSO-d_6) δ 7.97 (d, J=8.4, 2H), 7.87 (d, J=8.4, 2H), 7.66 (d, J=8.7, 1H), 7.48 (s, 2H), 6.97-7.04 (m, 2H). MS (ESI) 319.8 [M-H]$^-$. IR (cm^{-1}) 3383 and 3361 (NH), 1717 (C=O). Anal. Calcd. for: $C_{13}H_{11}N_3O_5S$, C, 48.60; H, 3.45; N, 13.08; S, 9.98. Found: C, 48.40; H, 3.49; N, 13.04; S, 9.94.

4.2.3 Synthesis of Compounds 10, 11 and 12

(*S*)-Methyl 3-phenyl-2-(4-(*N*-thiazol-2-ylsulfamoyl)benzamido)propanoate (22a). 4-Chlorosulfonyl benzoic acid (5.0 g, 22.7 mmol) was dissolved in a mixture of $SOCl_2$ (20 mL) and 1,2-dichloroethane (10 mL) and refluxed for 1 h. The solvent was removed under vacuum to give a light-brown solid. The solid was dissolved in anhydrous CH_2Cl_2 (25 mL) followed by the addition of L-phenylalanine methyl ester hydrochloride (5.14 g, 23.8 mmol) in anhydrous CH_2Cl_2 (25 mL) containing DIEA (4.0 mL) at –20 °C. The mixture was stirred for 30 min at –15 °C to 0 °C and then at RT for another 1 h. It was washed with $NaHCO_3$ solution, brine, dried ($MgSO_4$), and concentrated to give a solid, which was added to a solution of 2-aminothiazole (3.5 g, 34.59 mmol) in pyridine (20 mL). After stirring for 17 h, the solution was poured into 2 N HCl and extracted with EtOAc (3 x 50 mL). The combined extract was washed successively with water and brine, dried ($MgSO_4$), concentrated, and purified by flash chromatography (acetone/hexane, 4:3) to give a white solid in 40% yield. m.p. 95-99 °C. ^{1}H NMR (DMSO-d_6) δ 12.85 (s, 1H), 9.04 (d, J=7.5, 1H), 7.88 (s, 4H), 7.19-7.28 (m, 6H), 6.86 (d, J=3.3, 1H), 4.63-4.68 (m, 1H), 3.64 (s, 3H), 3.03-3.21 (m, 2H). MS (ESI) 443.9 [M-H]$^-$. IR (cm^{-1}) 3102 (NH), 1736 and 1643 (C=O).

(*S*)-*N*-(1-Hydroxy-3-phenylpropan-2-yl)-4-(*N*-thiazol-2-ylsulfamoyl)benzamide (23a). Compound **22a** (3.8 g, 8.5 mmol) was reduced to **23a** as described under general procedure 2. The crude product was purified by flash chromatography (CH_2Cl_2/acetone, 2:1) to give a white solid in 54.8% yield. m.p. 147-155 °C. ^{1}H NMR (DMSO-d^6) δ 12.84 (s, 1H), 8.35 (d, J=8.4, 1H), 7.87 (dd, J=8.4, 14.4, 4H), 7.12-7.28 (m, 6H), 6.86 (d, J=4.5, 1H), 4.85 (t, J=5.4, 1H), 4.10-4.19 (m, 1H), 3.40-3.52 (m, 2H), 2.94 (dd, J=5.1, 13.8, 1H), 2.77 (dd, J=9.0, 13.8, 1H). MS (ESI) 416.0 [M-H]$^-$. IR (cm^{-1}) 3260 and 3104 (NH and OH), 1639 (C=O).

(*S*)-*N*-(1-Oxo-3-phenylpropan-2-yl)-4-(*N*-thiazol-2-ylsulfamoyl)benzamide (10). Oxidation of **23a** (1.0 g, 2.4 mmol) with $PySO_3$ complex as described under general procedure 4 and purification of the crude product by flash chromatography (CH_2Cl_2/acetone, 2:1) gave 10 as a white solid in 52.25% yield. m.p.103-107 °C. ^{1}H NMR (DMSO-d_6) δ 12.84 (s, 1H), 9.61 (s, 1H), 9.06 (d, J=7.5, 1H), 7.81-7.94 (m,

4H), 7.15-7.30 (m, 6H), 6.86 (d, J=4.5, 1H), 4.50-4.57 (m, 1H), 3.28 (dd, J=5.2, 13.8, 1H), 2.91 (dd, J=10.5, 13.8, 1H). MS (ESI) 413.9 $[M-H]^-$, 446.0 $[M+MeOH-H]^-$. IR (cm^{-1}) 3313 (NH), 1731 and 1642 (C=O). Anal. Calcd. for: $C_{19}H_{17}N_3O_4S_2 \bullet 0.5H_2O$, C, 53.76; H, 4.27; N, 9.90; S, 15.11. Found: C, 53.98; H, 4.53; N, 9.58; S, 15.17.

(*S*)-Methyl 3-phenyl-2-(3-(*N*-thiazol-2-ylsulfamoyl)benzamido)propanoate (22b) 3-Chlorosulfonyl benzoic acid (7.0 g, 31.7 mmol) was dissolved in a mixture of $SOCl_2$ (28 mL) and 1,2-dichloroethane (14 mL) and refluxed for 1.5 h. The solvent was removed under vacuum and the residue was dissolved in anhydrous CH_2Cl_2 (35 mL) followed by the addition of L-Phe-OMe hydrochloride (7.18 g, 33.3 mmol) in anhydrous CH_2Cl_2 (35 mL) containing DIEA (5.8 mL) at –20 °C. The mixture was stirred for 30 min at –15 °C to 0 °C and at RT for another 1 h. The mixture was then washed with $NaHCO_3$ solution, brine, and dried ($MgSO_4$). The solvent was removed under vacuum and the product was recovered and added to a solution of 2-aminothiazole (3.49 g, 34.87 mmol) in pyridine (30 mL). After stirring for 17 h at RT, the mixture was poured into 2 N HCl and extracted with EtOAc (4 x 70 mL). The combined organic layer was washed successively with water and brine, dried ($MgSO_4$), concentrated, and purified by flash chromatography (acetone/hexane, 1:1) to give a white solid in 41% yield. m.p. 149-152 °C. ^{1}H NMR (acetone-d_6) δ 8.26-8.31 (m, 2H), 8.00 (d, J=7.8, 2H), 7.59 (t, J=7.8, 1H), 7.17-7.34 (m, 6H), 6.81 (d, J=4.5, 1H), 4.88-4.95 (m, 1H), 3.69 (s, 3H), 3.29 (dd, J=5.4, 13.8, 1H), 3.16 (dd, J=9.3, 13.8, 1H). MS (ESI) 443.9 $[M-H]^-$. IR (cm^{-1}) 3102 (NH), 1738 and 1648 (C=O).

(*S*)-*N*-(1-Hydroxy-3-phenylpropan-2-yl)-3-(*N*-thiazol-2-ylsulfamoyl)benzamide (23b). Compound **22b** (3.2 g, 7.2 mmol) was reduced as described under general procedure 2 followed by flash chromatographic purification (acetone/hexane, 3:2) to give **23b** as light yellow in solid 83.37% yield. m.p. 132-140 °C. ^{1}H NMR (DMSO-d_6) δ 12.80 (s, 1H), 8.47 (d, J=8.4, 1H), 8.22 (s, 1H), 7.98 (d, J=7.8, 1H), 7.91 (d, J=7.8, 1H), 7.61 (t, J=7.8, 1H), 7.15-7.28 (m, 6H), 6.86 (d, J=4.5, 1H), 4.85 (t, J=5.4, 1H), 4.16-4.18 (m, 1H), 3.42-3.54 (m, 3H), 2.95 (dd, J=5.1, 13.8, 1H), 2.79 (dd, J=9.3, 13.8, 1H). MS (ESI) 416.0 $[M-H]^-$. IR (cm^{-1}) 3316 (NH and OH), 1637 (C=O).

(*S*)-*N*-(1-Oxo-3-phenylpropan-2-yl)-3-(*N*-thiazol-2-ylsulfamoyl)benzamide (11). Oxidation of **23b** (500 mg, 1.199 mmol) with $PySO_3$complex as described under general procedure 4 and purification of the crude product by recrystallization from acetone/hexane to give **11** as a white solid in 50.6% yield. m.p. 180-182 °C. ^{1}H NMR (DMSO-d_6) δ 12.81 (s, 1H), 9.62 (s, 1H), 9.18 (d, J=7.5, 1H), 8.26 (t, J=1.5, 1H), 7.94-8.02 (m, 2H), 7.65 (t, J=7.8, 1H), 7.17-7.29 (m, 6H), 6.87 (d, J=4.5, 1H), 4.51-4.58 (m, 1H), 2.89-3.27 (m, 2H). MS (ESI) 414.0 $[M-H]^-$. IR (cm^{-1}) 3248 (NH), 1732 and 1637 (C=O). Anal. Calcd. for: $C_{19}H_{17}N_3O_4S_2 \bullet 0.25H_2O$, C, 54.34; H, 4.20; N, 10.00; S, 15.27. Found: C, 54.08; H, 4.38; N, 9.81; S, 15.35.

Methyl 2-(*N*-thiazol-2-ylsulfamoyl)benzoate (25). Methyl (2-sulfonyl chloride) benzoate (4 g, 17.0 mmol) was added to a solution of 2-aminothiazole (1.88 g, 18.7 mmol) in pyridine (20 mL) and stirred for 100 h at RT followed by heating at 55 °C for another 2 h. The mixture was poured into 4 N HCl and extracted with EtOAc (3 x 25 mL). The combined extract was washed successively with water and brine, dried ($MgSO_4$), concentrated, and purified by flash chromatography (ethyl acetate/hexane, 3:2) to give a brown solid in 78.7% yield. m.p. 202-205 °C. ^{1}H NMR (DMSO-d_6) δ 12.78 (s, 1H), 7.91-7.94 (m, 1H), 7.63-6.68 (m, 2H), 7.51-7.54 (m, 1H), 7.28 (d, J=4.8, 1H), 6.86 (d, J=4.5, 1H), 3.75 (s, 3H). MS (ESI) 321.1 $[M+H]^+$, IR (cm^{-1}) 3135 (NH), 1726 (C=O).

(*S*)-*N*-(1-Hydroxy-3-phenylpropan-2-yl)-2-(*N*-thiazol-2-ylsulfamoyl)benzamide (23c). Compound **25** (233 mg, 78.2 mmol) and 2-amino-3-phenyl-propanol (177 mg, 1.17 mmol) were dissolved in a mixture of DMF (2 mL), 1,4-dioxane (3 mL), and 2 drops TEA and heated in a microwave at 155 °C, 100 w and 100 psi for 20 min. The solvent was removed in vacuo and a few drops of 0.5 N HCl were added followed by recrystallization from acetone/hexane to give a light yellow solid in 35.8% yield. m.p. 99-109 °C. ^{1}H NMR (DMSO-d_6) δ 12.63 (s, 1H), 8.02 (d, J=8.4, 1H), 7.88-7.91 (m, 1H), 7.51-7.61 (m, 2H), 7.14-7.33 (m, 7H), 6.85 (d, J=4.5, 1H), 4.57 (br, 1H), 4.03-4.12 (m, 1H), 3.39-3.58 (m, 2H), 2.91 (dd, J=6.0, 13.5, 1H), 2.77 (dd, J=8.4, 13.8, 1H). MS (ESI) 416.0 $[M-H]^-$, 440.1 $[M+Na]^+$. IR (cm^{-1}) 3583, 3419 and 3258 (NH and OH), 1650 (C=O).

(*S*)-*N*-(1-Oxo-3-phenylpropan-2-yl)-2-(*N*-thiazol-2-ylsulfamoyl)benzamide (12). Oxidation of **23c** (460 mg, 1.10 mmol) with $PySO_3$ complex as described under general procedure 4 followed by flash chromatographic (ethyl acetate/CH_2Cl_2, 3:1) purification gave **12** as a white solid in 55.16% yield. m.p. 119-121 °C. ^{1}H NMR (acetone-d_6) δ 9.82 (s, 1H), 7.86-8.21 (m, 2H), 7.18-7.64 (m, 9H), 6.80-7.03 (m, 1H), 4.50-4.57 (m, 1H), 2.56-3.31 (m, 2H). MS (ESI) 414.1 $[M-H]^-$, 445.9 $[M+MeOH-H]^-$. IR (cm^{-1}) 3315 (NH), 1731 (CHO), 1647 (C=O). Anal. Calcd. for: $C_{19}H_{17}N_3O_4S_2 \bullet 0.5H_2O$, C, 53.76; H, 4.27; N, 9.90; S, 15.11. Found: C, 53.55; H, 4.37; N, 9.64; S, 15.43.

4.2.4 Synthesis of Compounds 13 and 14

(*S*)-Methyl 2-(4-(chlorosulfonyl)benzamido)-4-methylpentanoate (26a). A solution of 4-chlorosulfonyl benzoic acid (7.0 g, 31.7 mmol) in 1,2-dichloroethane (14 mL) and $SOCl_2$ (30 mL) was reflux for 1.5 h and the solvent was removed in vacuo to give a light-brown solid. The solid was dissolved in anhydrous CH_2Cl_2 (40 mL) followed by the addition of L-leucine methyl ester hydrochloride (8.64 g, 47.59 mmol) in anhydrous CH_2Cl_2 (30 mL) containing DIPEA (8.0 mL). The mixture was cooled at –20 °C and stirred for 30 min. The temperature was allowed to rise to RT and stirred for another 1.5 h. The crude product was purified by column

chromatography (ethyl acetate/hexane 1:3) to give a light yellow liquid in 90% yield. ^{1}H NMR ($CDCl_3$) δ 7.97-8.12 (m, 4H), 6.78 (d, J=8.1, 1H), 4.83-4.89 (m, 1H), 3.80 (s, 3H), 1.66-1.81 (m, 3H), 1.0 (t, J=5.4, 6H). MS (ESI) 346.2 $[M-H]^-$. IR (cm^{-1}) 1646.75 (amide), 1740.66 (ester).

(*S*)-Methyl 4-methyl-2-(4-(*N*-thiazol-2-ylsulfamoyl)benzamido)pentanoate (27a). 2-Aminothiazole (3.8 g, 38.0 mmol) and K_2CO_3 (10.5 g, 76.0 mmol) were added to a solution of **26a** (8.8 g, 25.3 mmol) in dry Acetonitrile (60 mL) and the mixture was stirred for 8 h at 75 °C, filtered, and the solvent was removed in vacuo. The solid was purified by flash chromatography (acetone/hexane, 3:2) to give a white solid in 43.5% yield. m.p. 85-89 ºC. ^{1}H NMR ($CDCl_3$) δ 7.897 (dd, J=8.7, 18.3, 4H), 7.174 (d, J=6.0, 1H), 6.864 (d, J=8.4, 1H), 6.581 (d, J=6.0, 1H), 4.814-4.887 (m, 1H), 3.785 (s, 3H), 1.683-1.806 (m, 3H), 0.993 (dd, J=3, 6, 6H). MS (ESI) 410.2$[M-H]^-$. IR (cm^{-1}) 16385 (amide), 1737 (ester).

(3*S*)-Methyl 2-hydroxy-3-((*S*)-4-methyl-2-(4-(*N*-thiazol-2-ylsulfamoyl) benzamido)-pentanamido)-4-phenylbutanoate (29a). A mixture of 1.0 N NaOH (30 mL) and MeOH (10 mL) was added to a solution of **27a** (420 mg, 1.02 mmol) in CH_2Cl_2 (50 mL) and stirred at RT for 3 h. The organic solvent was removed and the aqueous phase was adjusted to pH 2 with 5.0 N HCl. The solid that separated out was recovered and used in the next step without purification. The solid (500 mg, 1.26 mmol) was dissolved in anhydrous DMF (5 mL) and HBTU (0.45 g, 1.19 mmol) was added at 0 ºC. After stirring at RT for 1 h, **28**[167] (0.29 g, 1.38 mmol) and DIPEA (0.33 mL) in anhydrous DMF (5 mL) was added and the mixture was stirred for 16 h to give a white solid in 44% yield. m.p. 202-204 ºC . ^{1}H NMR (DMSO-d_6) δ 8.41 (dd, J=8.7, 12.3, 1H), 7.76-7.90 (m, 4H), 7.13-7.20 (m, 5H), 6.99 (d, J=3.9, 1H), 6.53 (d, J=3.9, 1H),4.38-4.46 (m, 1H), 4.22-4.30 (m, 1H), 3.99-4.08 (m, 1H), 3.49 (d, J=4.8,3H), 2.86 (dd, J=6.0, 13.2, 1H), 2.68 (dd, J=9.0, 13.2, 1H), 1.38-1.54 (m, 2H), 1.28-1.34 (m, 1H), 0.79-0.90 (m, 6H). MS (ESI) 587.2 $[M-H]^-$. IR (cm^{-1}) 1647.55(amide), 1737.95 (ester), 3306.25 (NH and OH).

(*S*)-Methyl 3-((*S*)-4-methyl-2-(4-(*N*-thiazol-2-ylsulfamoyl)benzamido) pentanamido)-2-oxo-4-phenylbutanoate (13). Dess-Martin oxidation (general procedure 3) of **29a** (350 mg, 0.59 mmol) followed by flash chromatographic (acetone/hexane, 1:1) purification gave **13** as a white solid in 53.3% yield. m.p. 118-122 ºC. ^{1}H NMR ($CDCl_3$) δ 12.50 (s, 1H), 7.70-7.78 (m, 4H), 7.52 (d, J=8.4, 1H), 7.06-7.38 (m, 6H), 6.58 (dd, J=0.9, 4.5, 1H), 5.26-5.36 (m, 1H), 4.72 (dd, J=8.4, 14.1, 1H), 3.79 (d, J=3.3, 3H), 3.26 (dt, J=5.7, 13.8, 1H), 3.00 (dt, J=8.7, 14.1, 1H), 1.45-1.74 (m,3H), 0.83-0.94 (m, 6H). MS (ESI) 585.0 [M-H]-. IR (cm-1) 1632 (amide and α-ketone), 1732 (ester), 3298 (NH). Anal. Calcd. for: $C_{27}H_{30}N4O_7S_2$, C, 55.28; H, 5.15; N, 9.55; S, 10.93. Found: C, 55.08; H, 5.41; N, 9.36; S, 10.64.

(*S*)-Methyl 4-methyl-2-(3-(*N*-thiazol-2-ylsulfamoyl)benzamido)pentanoate (27b). $SOCl_2$ (30 mL) was added to an ice-cooled solution of 3-chlorosulfonyl

benzoic acid (7.0 g, 31.7 mmol) in 1,2-dichloroethane (14 mL) at 0 ºC. The mixture was allowed to warm to RT and then refluxed for 2 h. The solvent was removed in vacuo to give a light-brown liquid, which was dissolved in anhydrous CH_2Cl_2 (30 mL) and L-leucine methylester hydrochloride (8.64 g, 47.59 mmol) in anhydrous CH_2Cl_2 (30 mL) containing DIEA (8 mL) was added at –20 ºC. The mixture was stirred for 30 min. at –15 ºC to 0 ºC and then at RT for 1.5 h. The solvent was removed to give an oil (91.8%), which was used in the next step without purification. The oil (6.8 g, 19.6 mmol) was added to a solution of 2-aminothiazole (1.96 g, 19.6 mmol) in CH_2Cl_2 (50 mL) containing anhydrous pyridine (35 mL). The mixture was heated at 40 ºC for 58 h and the solvent was removed in vacuo. The resulting gummy solid was dissolved in EtOAc containing 2 N HCl. The organic phase was separated, washed with 0.5 N HCl, brine, and dried ($MgSO_4$). The solvent was evaporated and the residue was purified by column chromatography (acetone/hexane, 4:3) to give colorless oil in 44.4% yield. ^{1}H NMR ($CDCl_3$) δ 8.27 (s, 1H), 7.88 (d, J=7.8, 2H), 7.44 (d, J=7.8, 1H), 7.37 (t, J=8.4, 1H), 7.04 (d, J=4.8, 1H), 6.49 (d, J=4.8, 1H), 4.72-4.75 (m, 1H), 3.66 (s, 3H), 1.64-1.66 (m, 3H), 0.86 (d, J=5.7, 6H). MS (ESI) 410.0 $[M-H]^-$. IR (cm^{-1}) 3298 (NH), 1744 (C=O).

(3S)-Methyl 2-hydroxy-3-((*S*)-4-methyl-2-(3-(*N*-thiazol-2-ylsulfamoyl) benzamido)-pentanamido)-4-phenylbutanoate (29b). NaOH (2.0 N, 75 mL) was added to a solution of **27b** (3.4 g, 8.27 mmol) in MeOH (150 mL) and the mixture was stirred at RT for 1.5 h. The MeOH was removed and the aqueous phase was adjusted to pH 2 with 5.0 N HCl to give a light yellow solid (3.0 g), which was used without purification. Mukayaima's reagent (1.79 g, 7.0 mmol) in anhydrous DMF (8 mL) was added into a stirred ice-cooled solution of above solid (2.54 g, 6.4 mmol), **28**[167] (1.21 g, 5.8 mmol) and TEA (2 mL) in anhydrous DMF (11 mL). After stirring at RT for 36 h, the solution was poured into H_2O (200 mL) and the white solid that separated out was recovered by filtration and the filtrate was extracted with EtOAc (3 x 25 mL). Evaporation of the solvent gave more solid and the combined solids were purified by column chromatography (CH_2Cl_2/MeOH, 20:1) to give a white solid in 31.9% yield. ^{1}H NMR (acetone-d_6) δ 8.39 (t, J=1.5, 1H), 8.09 (dt, J=1.5, 7.8, 1H), 8.01-8.04 (m, 2H), 7.608 (t, J=7.8, 1H), 7.08-7.36 (m, 6H), 0.79 (d, J=4.5, 1H), 4.61-4.68 (m, 1H), 4.42-4.51 (m, 1H), 4.15 (d, J=2.4, 1H), 3.56-3.64 (m, 3H), 1.55-1.73 (m, 3H), 0.82-0.99 (m, 6H). MS (ESI) 587.0 $[M-H]^-$. IR (cm^{-1}) 3317 (NH, OH), 1736 and 1639 (C=O).

***N*-((2*S*)-1-((2*S*)-4-Amino-3-hydroxy-4-oxo-1-phenylbutan-2-ylamino)-4-methyl-1-oxopentan-2-yl)-3-(*N*-thiazol-2-ylsulfamoyl)benzamide (30).** Compound **29b** (700 mg, 1.19 mmol) was dissolved in 7.0 N methanolic ammonia (35 mL) and stirred at RT for 48 h following which the solvent was removed and the product was purified by column chromatography (acetone/hexane/MeOH, 6:2:1) to give a white solid in 69.6% yield. m.p. 151-157 ºC. ^{1}H NMR (DMSO-d_6) δ 8.68-8.74 (m, 1H), 8.30 (t, J=1.5, 1H), 8.08 (d, J=7.8, 1H), 8.95 (dt, J=7.8, 0.9, 1H), 7.57-7.71 (m, 2H), 7.02-7.25 (m, 8H), 6.08 (d, J=4.5, 1H), 5.95 and 5.78 (s, 2:1, 1H), 4.41-4.53 (m, 1H),

4.17-4.25 (m,1H), 3.83 and 3.75 (s, 1:2, 1H), 2.80-2.88 (m, 1H), 2.58-2.68 (m, 1H), 1.34-1.64 (m, 3H), 0.81-0.96 (m, 6H). MS (ESI) 572.1 $[M-H]^-$. IR (cm^{-1}) 3298 (OH and NH), 1645 (C=O).

***N*-((*S*)-1-((*S*)-4-Amino-3,4-dioxo-1-phenylbutan-2-ylamino)-4-methyl-1-oxopentan-2-yl)-3-(*N*-thiazol-2-ylsulfamoyl)benzamide (14).** Oxidation of **30** (425 mg, 0.742 mmol) with pyridine-sulfur trioxide complex as described under general procedure 4 followed by flash chromatographic purification (acetone/hexane, 3:2) of the crude product gave **14** as a white solid in 54.9% yield. m.p 228-231 ºC. ^1H NMR (DMSO-d_6) δ 12.80 (s, 1H), 8.72 (d, J=8.4, 1H), 8.29-8.35 (m, 2H), 8.06 (t, J=7.8, 2H), 7.95 (d, J=8.1, 1H), 7.79 (s, 1H), 7.63 (t, J=7.8, 1H), 7.12-7.27 (m, 6H), 6.85 (dd, J=1.5, 4.5, 1H), 5.18-5.25 (m, 1H), 4.53-4.60 (m, 1H), 3.11 (dd, J=4.2, 14.4, 1H), 2.83 (dd, J=9.3, 14.4, 1H), 1.49-1.67 (m, 3H), 0.80-0.91 (m, 6H). MS (ESI) 570.0 $[M-H]^-$. IR (cm^{-1}) 3426 and 3276 (NH), 1731, 1674 and 1637 (C=O). Anal. Calcd. for: $C_{26}H_{29}N_5O_6S_2$, C, 54.63; H, 5.16; N, 12.02; S, 11.22. Found: C, 54.27; H, 5.16; N, 12.02; S, 10.99

4.2.5 Synthesis of Compounds 15-17

(*S*)-3-Methyl-2-(4-(phenyldiazenyl)phenylsulfonamido)butanoic acid (32). 4-Phenyl azobenzenesulfonyl chloride (3 g, 10.69 mmol) was added to a stirred ice-cooled solution of L-H-Val-OMe·HCl (2.15 g, 12.8 mmol) in anhydrous pyridine (15 mL) and the resulting crimson solution was stirred at RT for 44 h. Following this the mixture was poured into 2.0 N HCl (100 mL) and extracted with EtOAc (2 x 60 mL). The extract was washed successively with 1.0 N HCl, saturated $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (EtOAc/hexane, 1:2) to give (*S*)-Methyl-3-methyl-2-(4-(phenyldiazenyl)phenyl-sulfonamido)butanoate as an orange solid in 92.5% yield. m.p. 150-152 ºC. ^1H NMR ($CDCl_3$) δ 7.94-8.03 (m, 6H), 7.53-7.60 (m, 3H), 5.18 (d, J=9.9, 1H), 3.83 (dd, J_1=10.2, 5.1, 1H), 3.47 (s, 3H), 2.04-2.11 (m, 1H), 0.99 (d, J=6.9, 3H), 0.90 (d, J=6.9, 3H). MS (ESI) 374.0 $[M-H]^-$, 398 $[M+Na]^+$. IR (cm^{-1}) 1736 (ester), 3282 (amide). NaOH (1.0 N, 100 mL) was added to a solution of the orange solid (3.3 g, 8.8 mmol) in MeOH (50 mL) and the mixture was stirred at 55 ºC for 4 h after which the pH was adjusted to 2 with 2.0 N HCl. The solvent was removed and the crude product was recrystallized from CH_2Cl_2/ethyl acetate to give **32** as a yellow solid in 75.5% yield. m.p.220-222 ºC. ^1H NMR ($CDCl_3$) δ 7.95-8.00 (m, 6H), 7.54-7.59 (3H), 5.17 (d, J=10.2, 1H), 3.87 (dd, J=4.5, 9.9, 1H), 2.41 (br, 1H, COOH), 2.07-2.18 (m, 1H), 0.99 (d, J=6.6, 3H), 0.88 (d, J=6.6, 3H). MS (ESI) 360.0 $[M-H]^-$, 384.1 $[M+Na]^+$. IR (cm^{-1}) 1694 (C=O), 2876 (br, COOH), 3294 (amide).

(*S*)-3-Methyl-*N*-((*S*)-4-methyl-1-oxopentan-2-yl)-2-(4-(phenyldiazenyl)phenylsulfonamido)-butanamide (15). CDI (494 mg, 3.05 mmol) was added to a stirred

ice-cooled solution of **32** (1.0 g, 2.77 mmol) in a mixture of THF (15 mL) and CH_2Cl_2 (20 mL). Stirring was continued at RT for 30 min. and L-leucinol (531µL, 4.16mmol) was added and the mixture was stirred at RT for 96 h. The solvent was removed and the residue was recrystallized from acetone/CH_2Cl_2 mixture to give (*S*)-*N*-((*S*)-1-hydroxy-4-methylpentan-2-yl)-3-methyl-2-(4-(phenyldiazenyl)-phenyl-sulfonamido)butanamide as a yellow solid in 51.0% yield. m.p.237-238 ºC. ^{1}H NMR (acetone-d_6) δ 7.98-8.08 (m, 6H), 7.62-7.68 (m, 3H), 6.98 (d, J=8.1, 1H), 6.52 (d, J=8.7, 1H), 3.69-3.81 (m, 3H), 3.36-3.42 (m, 2H), 2.00-2.06 (m, 1H), 1.10-1.19 (m, 3H), 1.00 (d, J=6.9, 3H), 0.89 (d, J=6.9,3H), 0.69 (d, J=6.0, 3H), 0.64 (d, J=6.0, 3H). MS (ESI) 459.0 $[M-H]^-$, 483.3 $[M+Na]^+$. IR (cm^{-1}) 1639 (C=O), 3284 (amide), 3505 (amide). Dess-Martin oxidation (general procedure 3) of the yellow solid (300 mg, 0.65 mmol) followed by flash chromatographic purification (acetone /CH_2Cl_2/hexane, 2:1:4) gave **15** in 50.2% yield. m.p. 146-149 ºC. ^{1}H NMR (acetone-d_6) δ 9.50 and 9.42 (s, 2:1, 1H)), 7.94-8.10 (m, 6H), 7.54-7.63 (m, 3H), 6.30 and 6.01 (d, 1:2, J=7.2 and 7.8, 1H), 5.45 and 5.26 (d, 2:1, J=8.7 and 8.1, 1H), 4.40-4.48 (m, 1H), 3.63-3.70 (m, 1H), 2.09-2.20 (m, 1H), 1.19-1.70 (m, 3H), 0.79-1.01 (m, 12H). MS (ESI) 457.0 $[M-H]^-$, 513.3 $[M+Na+MeOH]^+$. IR(cm^{-1}) 1715 (CHO), 1668 (C=O). Anal. Calcd. for: $C_{23}H_{30}N_4O_4S$, C, 60.24; H, 6.59; N, 12.22; S, 6.99. Found: C, 60.05; H, 6.66; N, 12.17; S, 6.83.

(2*S*)-*N*-((2*S*)-1-Cyano-1-hydroxy-4-methylpentan-2-yl)-3-methyl-2-(4-(phenyldiazenyl)-phenylsulfonamido)butanamide (34). $NaHSO_3$ (3.2%, 11 mL) was added to a solution of **15** (847 mg, 1.85 mmol) in MeOH (30 mL) and stirred at RT for 30 h. EtOAc (25 mL) was added followed by 3.2% KCN solution and stirring was continued for another 5 h. The EtOAc layer was separated and the aqueous layer was extracted twice with EtOAc. The combined organic layer was washed with water, brine, and dried ($MgSO_4$). The solvent was removed in vacuo to give an orange solid in 90% yield. m.p 93-98 ºC. ^{1}H NMR (CDCl3) δ 7.96-8.04 (m, 6H), 7.56-7.63 (m, 3H), 6.40-6.53 (m, 1H), 5.43-5.52 (m, 1H), 4.50-4.57 (m, 1H), 4.08-4.24 (m, 1H), 3.55-3.66 (m, 1H), 2.12-2.24 (m, 1H), 1.40- 1.58 (m, 3H), 0.74-1.00 (m, 12H). MS (ESI) 508.1 $[M+H]^+$. IR (cm^{-1}) 3322 (OH), 1659 (C=O)

(3*S*)-Methyl-2-hydroxy-5-methyl-3-((*S*)-3-methyl-2-(4-(phenyldiazenyl) phenylsulfonamido)-butanamido)hexanoate (35). Conc. HCl (15 mL) was added to a solution of **34** (810 mg, 1.67 mmol) in MeOH (20 mL) and the mixture was stirred at 62 ºC for 24 h followed by concentration in vacuo and purification by column chromatography (EtOAc/hexane, 1:1) to give an orange solid in 69.4% yield. m.p 163-168 ºC. ^{1}H NMR ($CDCl_3$) δ 7.95-8.02 (m, 6H), 7.55-7.57 (m, 3H), 5.96 (d, J=9.0, 1H), 5.57 (d, J=7.8, 1H), 4.22-4.33 (m, 2H), 3.80 (s, 3H), 3.59 (dd, J=4.8, 7.8, 1H), 2.07-2.15 (m, 1H), 1.04-1.41 (m, 3H), 0.68-0.98 (m, 12H). MS (ESI) 517.0 $[M-H]^-$, 541.2 $[M+Na]^+$. IR (cm^{-1}) 3522, 3355 and 3270 (NH and OH), 1737 and 1648 (C=O).

(3*S*)-2-Hydroxy-5-methyl-3-((*S*)-3-methyl-2-(4-(phenyldiazenyl)phenylsulfonamido)-butanamido)hexanamide (36). Compound **35** (600 mg, 1.15 mmol) was

dissolved in 7.0 N methanolic ammonia (30 mL) and stirred at RT for 48 h following which the solvent was removed and the product was purified by column chromatography (ethyl acetate/hexane, 2:1) to give an orange solid in 68.8% yield. m.p 243-248 ºC. ^{1}H NMR (DMSO-d_6) δ 7.39-8.06 (m, 11H), 7.03-7.14 (m, 2H), 5.52 (dd, J=5.7, 15.3, 1H), 3.93-3.96 (m, 1H), 3.64-3.74 (m, 2H), 1.85-1.92 (m, 1H), 1.23-1.26 (m, 1H), 0.70-1.11 (m, 8H), 0.43-0.63 (m, 6H). MS (ESI) 501.9 $[M-H]^-$, 526.2 $[M+Na]^+$. IR (cm^{-1}) 3454, 3357 and 3322 (NH and OH), 1668 and 1644 (C=O).

(1*S*)-*N*-((*S*)-1-Amino-5-methyl-1,2-dioxohexan-3-yl)-2-(4-(phenyldiazenyl) phenylsulfonyl)-cyclopentane carboxamide (17). Oxidation of **36** (350 mg, 0.696 mmol) with pyridine-sulfur trioxide complex as described under general procedure 4 followed by column chromatographic purification (acetone/hexane 1:1) gave **17** as an orange solid in 71.7% yield. m.p. 134-136 ºC. ^{1}H NMR (acetone-d_6) δ 7.97-8.09 (m, 6H), 7.61-7.68 (m, 2H), 7.46 (d, J=6.9, 2H), 6.99 (s, 1H), 6.56 (d, J=9.3, 1H), 5.03-5.10 (m, 1H), 1.19-1.52 (m, 3H), 1.00 (d, J=6.9, 3H), 0.89 (d, J=6.9, 3H), 0.73 (dd, J=6.3, 9.3, 6H). MS (ESI) 500.6 $[M-H]^-$. IR (cm^{-1}) 3464, 3360 and 3281 (NH), 1736 (C=O), 1693 (C=O). Anal. Calcd. for: $C_{24}H_{31}N_5O_5S\bullet0.5H_2O$, C, 56.45; H, 6.32; N, 13.72; S, 6.28. Found: C, 56.30; H, 6.09; N, 13.59; S, 6.16.

(1*S*)-2-(4-(Phenyldiazenyl)phenylsulfonyl)cyclopentanecarboxylic acid (33). 4-Phenyl azobenzenesulfonyl chloride (5 g, 17.8 mmol) was added to an ice-cooled solution of D-Pro-OMe·HCl (3.54 g, 21.3 mmol) in anhydrous pyridine (25 mL) and stirred for 20 h at RT after which the mixture was poured into 2.0 N HCl (50 mL) solution and extracted with EtOAc (2 x 40 mL). The EtOAc extract was washed successively with 2.0 N HCl, saturated $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (acetone/hexane, 1:2) to give (1*S*)-methyl-2-(4-(phenyldiazenyl)phenylsulfonyl) cyclopentane carboxylate as a yellow solid in 87.3% yield. m.p. 126-128 ºC . ^{1}H NMR ($CDCl_3$) δ 8.04 (s, 4H), 7.95-7.98 (m, 2H), 7.53-7.60 (m, 3H), 4.40 (dd, J=5.6, 6.9, 1H), 3.73 (s, 3H), 3.52-3.58 (m, 1H), 3.39-3.45 (m, 1H), 1.98-2.12 (m, 3H), 1.84-1.87 (m, 1H). MS (ESI) 396.1 $[M+Na]^+$. IR(cm^{-1}) 1743 (C=O). The yellow solid (1.5 g, 4 mmol) was dissolved in MeOH (50 mL) and 1.0 N NaOH (90 mL) was added and the mixture was stirred at RT for 16 h followed by 2 h of refluxing. The mixture was adjusted to pH 2 with 2.0 N HCl and extracted with EtOAc. The extract was washed with brine, dried ($MgSO_4$), concentrated and purified by recrystallization from CH_2Cl_2/hexane to give **33** as an orange solid in 87.3% yield. m.p. 174-177 ºC. ^{1}H NMR ($CDCl_3$) δ 8.05 (s, 4H), 7.96-7.99 (m, 2H), 7.55-7.57 (m, 3H), 4.63 (br, 1H), 3.58 (s, 1H), 3.35-3.40 (m, 1H), 1.83-2.18 (m, 4H). MS (ESI) 358.0 $[M-H]^-$. IR (cm^{-1}) 1730 (acid), 2883 (br, COOH).

(1*S*)-*N*-((*S*)-4-Methyl-1-oxopentan-2-yl)-2-(4-(phenyldiazenyl)phenylsulfo-nyl)cyclopentane-carboxamide (16). CDI (474 mg, 2.93 mmol) was added to an ice-cooled solution of **33** (955 g, 2.66 mmol) in a mixture of THF (20 mL) and CH_2Cl_2 (15 mL) and stirred for 30 min. at RT. L-Leucinol was added and the mixture

was stirred at RT for 70 h after which the solvent was removed and the residue was recrystallized from CH_2Cl_2/hexane to give (1*S*)-*N*-((*S*)-1-hydroxy-4-methylpentan-2-yl)-2-(4-(phenyldiazenyl)phenylsulfonyl)cyclopentane carboxamide as orange solid in 60.0% yield. m.p. 152-154 ºC. ^{1}H NMR ($CDCl_3$) δ 7.97-8.10 (m, 6H), 7.55-7.59 (m, 3H), 6.75 (d, J=9.0, 1H), 4.08-4.16 (m, 1H), 4.03 (dd, J=3.6, 8.4, 1H), 3.81 (dd, J=3.6, 11.4, 1H), 3.67-3.73 (m, 1H), 3.55 (dd, J=5.4, 11.4, 1H), 3.21-3.29 (m, 1H), 1.65-1.88 (m, 5H), 1.39-1.65 (m, 2H), 0.97 (t, J=6.3, 6H). MS (ESI) 481.2 $[M+Na]^+$. IR (cm^{-1}) 1636 (C=O), 3376 (amide). Dess-Martin oxidation (general procedure 3) of the orange solid (557 mg, 1.21 mmol) followed by column chromatographic purification (ethyl acetate/hexane, 2:1) of the crude product gave **16** as yellow solid in 50.4% yield. m.p. 79-82 ºC. ^{1}H NMR ($CDCl_3$) δ 9.63 (s, 1H), 7.98-8.13 (m, 6H), 7.57-7.60 (m, 3H), 7.26 (s, 1H), 4.43-4.50 (m, 1H), 4.22-4.25 (m, 1H), 3.61-3.68 (m, 1H), 3.25-3.34 (m, 1H), 2.24-2.30 (m, 1H), 2.24-2.30 (m, 1H), 1.66-1.86 (m, 6H), 1.01 (dd, J=6.0, 8.1, 6H). MS (ESI) 511.3 $[M+MeOH+Na]^+$. IR(cm^{-1}) 1655 (C=O), 1735 (CHO), 3366 (amide). Anal. Calcd. for: $C_{23}H_{28}N_4O_4S•0.5H_2O$, C, 59.34; H, 6.28; N, 12.03; S, 6.89. Found: C, 59.28; H, 6.16; N, 11.96; S, 6.69.

4.2.6 Synthesis of Compounds 18 and 19

(*S*)-(*E*)-Methyl 3-methyl-2-(4-styrylphenylsulfonamido)butanoate (39). Bromobenzene (12.2 mL, 116 mmol), $Pd(OAc)_2$ (387 mg, 1.74 mmol), TEA (17 mL), and $(o\text{-}Tol)_3P$ (1.06 g, 3.5 mmol) were added to a solution of p-styrene sulfonic acid sodium salt (20 g, 97 mmol) in DMF (200 mL)/ H_2O (4 mL) mixture. The mixture was stirred at 100 ºC for 2 h. After cooling to RT, a gray solid (21.9 g, 80%) separated out and was recovered by filtration, dried, and used in the next step without further purification. The solid was dissolved in (87 mL)/anhydrous DMF (4.5 mL) mixture and refluxed at 90 ºC for 3h. The solvent was removed in vacuo and the residue was dissolved in EtOAc (150 mL) and washed successively with 2.0 N HCl, saturated $NaHCO_3$, brine, and dried ($MgSO_4$). The EtOAc layer was recovered and concentrated under vacuum to give a gray solid (20.19 g, 93.3%), which was used without further purification. The solid (5g, 17.95 mmol) was mixed with L-Val-OMe hydrochloride (3.6 g, 21.47 mmol) and DIPEA (4 mL) in pyridine (25 mL) and the mixture was stirred for 4 days at RT, poured into 2.0 N HCl and extracted with EtOAc. The extract was washed twice with aqueous 1 N HCl, saturated aqueous $NaHCO_3$, brine, and dried ($MgSO_4$). The solvent was concentrated in vacuo and the residue was purified by column chromatography (acetone/hexane, 1:1) to give a white solid in 45% yield. m.p. 173-175 ºC. ^{1}H NMR (DMSO-d_6) δ 8.26 (d, J=9.3), 7.639-7.800 (m, 6H), 7.295-7.472 (m, 5H), 3.559 (dd, J=7.2, 9.0, 1H), 3.359 (s, 3H), 1.870-1.938 (m, 1H), 0.820 (dd, J=6.9, 9.0, 6H). MS (ESI) 372.0 $[M-H]^-$, 396.1 $[M+Na]^+$. IR (cm^{-1}) 1735 (ester), 3278 (NH).

(*S*)-(*E*)-3-Methyl-2-(4-styrylphenylsulfonamido)butanoic acid (40). NaOH (4.0 N, 40 mL) was added to a solution of **39** (6.85 g, 18.4 mmol) in MeOH (40 mL) and acetone (40 mL) mixture and stirred for 16 h at 48 ºC followed by adjustment of the pH to 2 with 2.0 N HCl and extraction of the solid that separated out with EtOAc. The extract was washed with brine, dried ($MgSO_4$), filtered, and concentrated. The residue was purified by recrystallization from acetone/hexane to give a yellow solid 80% yield. m.p. 193-195 ºC. ^{1}H NMR (DMSO-d_6) δ 12.58 (s, 1H), 8.01 (d, J=9.7), 7.75 (s, 4H), 7.65 (d, J=7.2, 2H), 7.29-7.46 (m, 5H), 3.54 (dd, J=6.0, 9.0, 1H), 1.90-1.97 (m, 1H), 0.81 (dd, J=6.9, 9.6, 6H). MS (ESI) 358.0 $[M-H]^-$. IR (cm^{-1}) 1747 (C=O), 3313 (w, COOH).

(*S*)-*N*-((*S*)-1-Hydroxy-4-methylpentan-2-yl)-3-methyl-2-(4-styrylphenylsulfonamido)-butanamide (41). L-Leucinol (2.51 mL, 19.2 mmol) and TEA (4.53 mL, 32.5 mmol) were added to a solution of **40** (5.3 g, 14.76 mmol) in anhydrous DMF (150 mL) at 0 ºC followed by the addition of Mukayaima's reagent (4.53 g, 17.7 mmol). After stirring for 4 days at RT, the solution was poured into H_2O (1.5 L) to give the crude product, which was purified by recrystallization from ethyl acetate to obtain a white solid in 47% yield. m.p. 196-197 ºC. ^{1}H NMR (DMSO-d_6) δ 7.62-7.79 (m, 7H), 7.54 (d, J=8.7, 1H), 7.28-7.43 (m, 5H), 4.55 (s, 1H), 3.48-3.56 (m, 1H), 3.04-3.19 (m, 2H), 1.80-1.86 (m, 1H), 1.10-1.18 (m, 2H), 0.99-1.04 (m, 1H), 0.63-0.86 (m, 12H). MS (ESI) 457.0 $[M-H]^-$, 481.3 $[M+Na]^+$. IR (cm^{-1}) 1638 (C=O), 3282 (sulfamide), 3205 (amide), 3503 (br, OH).

(*S*)-3-Methyl-*N*-((*S*)-4-methyl-1-oxopentan-2-yl)-2-(4-styrylphenylsulfonamido)butanamide (18). Oxidation of **41** (2.58 g, 5.63 mmol) with $PySO_3$ complex (general procedure 4) followed by recrystallization of the crude product from ethyl acetate/hexane gave **18** as a white solid in 85% yield. m.p. 174-175 ºC. ^{1}H NMR (DMSO-d_6) δ 9.51 (s, 1H), 7.85 (d, J=8.5, 2H), 7.62 (d, J=8.5, 2H), 7.42 (t, J= 8.0, 2H), 7.35 (t, J=7.5, 1H), 7.25 (d, J=16.5, 1H), 7.10 (d, J=16.5, 1H), 6.12 (d, J=7.5,1H), 5.34 (d, J=8.5, 1H), 4.41-4.45 (m, 1H), 3.62 (dd, J=5.0, 8.0, 1H), 2.10-2.17 (m, 1H), 1.54-1.58 (m, 1H), 1.41-1.49 (m, 1H), 1.26-1.32 (m, 1H), 0.96 (d, J=6.5, 3H), 0.85-0.90 (m, 9H). MS (ESI) 374.0 $[M-H]^-$, 398 $[M+Na]^+$. IR (cm^{-1}) 1637 (C=O), 1728 (CHO). Anal. Calcd. for: $C_{25}H_{32}N_2O_4S$, C, 65.76; H, 7.06; N, 6.14; S, 7.02. Found: C, 65.51; H, 7.10; N, 6.13; S, 7.24.

(2*S*)-*N*-((2*S*)-1-Cyano-1-hydroxy-4-methylpentan-2-yl)-3-methyl-2-(4-styrylphenyl-sulfonamido)butanamide (42). $NaHSO_3$ (3.2%, 14mL) was added to a solution of **18** (2 g, 4.38 mmol) in MeOH (30 mL) and stirred at RT for 40 h followed by the addition of 3.2% KCN solution (10 mL) and EtOAc (50 mL). After stirring for another 5 h, the mixture was extracted with EtOAc (3 x 50mL). The extract was concentrated and the residue was purified by recrystallization from acetone/hexane to give **19** as a white solid in 90% yield. m.p. 125-128 ºC. ^{1}H NMR ($CDCl_3$) δ 7.09-7.88 (m, 11H), 6.53-6.69 (m, 1H), 5.33-5.40 (m, 1H), 4.51-4.59 (m, 1H), 4.10-4.27 (m, 1H), 3.50-3.61 (m, 1H), 2.15-2.25 (m, 1H), 1.43-1.62 (m, 3H), 0.81-0.98 (m, 12H).

MS (ESI) 374.0 $[M-H]^-$, 398 $[M+Na]^+$. IR (cm^{-1}) 1736 (ester), 3282 (amide).

(3*S*)-Methyl-2-hydroxy-5-methyl-3-((*S*)-3-methyl-2-(4-styrylphenylsulfonamido)-butanamido) hexanoate (43). Compound **42** (932 mg, 19.4 mmol) was dissolved in MeOH (40 mL) containing conc. HCl (11 mL) and the mixture was stirred for 24 h at 54 ºC. The solvent was removed in vacuo and the residue was purified by column chromatography (acetone/hexane, 1:1) to give **43** as a white solid in 75.2% yield. m.p. 180-183 ºC. ^{1}H NMR (DMSO-d_6) δ 7.28-7.73 (m, 13H), 5.35-5.58 (m, 1H), 3.83-3.97 (m, 2H), 3.58-3.68 (m, 1H), 1.79-1.83 (m, 1H), 1.01-1.27 (m, 3H), 0.55-0.91 (m, 12H). MS (ESI) 374.0 $[M-H]^-$, 398 $[M+Na]^+$. IR (cm^{-1}) 1736 (ester), 3282 (amide).

(3*S*)-2-Hydroxy-5-methyl-3-((*S*)-3-methyl-2-(4-styrylphenylsulfonamido) butanamido)-hexanamide (44). A solution of **43** (750 mg, 1.4 mmol) in 7.0 N methanolic ammonia (30 mL) was stirred at RT for 96 h and the mixture was concentrated to give a residue, which was purified by column chromatography (acetone/hexane, 2:1) to obtain **44** as a white solid in 64% yield. m.p. 234-242 ºC. ^{1}H NMR (DMSO-d_6) δ 7.04-7.77 (m, 15H), 5.49-5.54(m, 1H), 3.96-4.03 (m, 1H), 3.58-3.71 (m, 2H), 1.84-1.93 (m, 1H), 1.24-1.34 (m, 1H), 1.03-1.15 (m, 2H), 0.53-0.87 (m, 12H). MS (ESI) 500.0 $[M-H]^-$. IR (cm^{-1}) 1660, 1643 (C=O), 3326 (OH).

(*S*)-5-Methyl-3-((*S*)-3-methyl-2-(4-styrylphenylsulfonamido)butanamido)-2-oxohexanamide (19). Oxidation of **44** (400 mg, 0.798 mmol) with pyridine-sulfur trioxide complex (general procedure 4) followed by flash chromatographic purification (acetone/hexane, 1:1) of the crude product gave **19** as a white solid in 52.7% yield. m.p. 209-211 ºC. ^{1}H NMR (DMSO-d_6) δ 8.12 (d, J=6.9, 1H), 7.97 (s, 1H), 7.63-7,77 (m, 8H), 7.29-7.45 (m, 5H), 4.79 (t, J=7.2, 1H), 3.62 (dd, J=6.6, 9.3, 1H), 1.81-1.88 (m, 1H), 1.14-1.33 (m, 3H), 0.63-0.89 (m, 12H). MS (ESI) 498.0$[M-H]^-$, 554.3 $[M+MeOH+Na]^+$. IR (cm^{-1}) 1637 (C=O), 1693 (C=O), 1740 (C=O) 3124, 3276, 3359, 3463 (amide). Anal. Calcd. for: $C_{26}H_{33}N_3O_5S\bullet0.2H_2O$, C, 62.06; H, 6.69; N, 8.35; S, 6.37. Found: C, 62.01; H, 6.59; N, 8.33; S, 6.27.

4.3 Determination of the Antiproliferative Activity of the Calpain Inhibitors

4.3.1 Cell Cultures

Cell lines were cultured in a Dulbecco's modified Eagle's Medium (DMEM, Cellgro Mediatech, Inc.), supplemented with 5% (v/v) fetal bovine serum (FBS, Atlanta Biologics, Inc.), 1% (v/v) antibiotic/antimycotic solution (Sigma), and 0.05% bovine insulin (25mM, pH 8.2, Sigma) for human A375 melanoma cells and mouse

B16F1 melanoma cells. Cell cultures were maintained at 37 ^{o}C in a humidified atmosphere which contained 5% CO_2.

4.3.2 Cell Death Assay and GI_{50} Value Determination in Melanoma Cells.

During the initial screen, compounds dissolved in DMSO were added to the plates to give a 20 μM solution followed by sulforhodamine B (SRB). To determine GI_{50} values, human melanoma cell line A375 and mouse melanoma cell line B16F1 were trypsinized and harvested by centrifugation for 3 minutes. Cells were then resuspended in the same media (5% FBS, 1% antibiotic and 0.05% insulin in DMEM) and counted using a hemocytometer. Cells were seeded into U-bottom 96-well microtiter plate at 5000 cells/well. After 12 h, media were changed and 8 dilutions of compounds were added. Cells were incubated with each compound for 48 h. Positive controls were performed with taxol. Negative controls were performed with cells incubated with media only.

Cell death was quantitated by sulforhodamine B (SRB) assay according to the manufacturer's protocol (Sigma-Aldrich).[172] Cells were fixed with 10% TCA, washed five times with tap water, and incubated with 0.4% sulforhodamine B in 1% acetic acid solution for 30 min at RT. 10 mM solution of Tris Base (Fluka) was added to release the dye from the cells for 1 h and the absorbance was measured at 490 nm in EL800 Absorbance Microplate reader (Biotek).

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