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Novel cell-penetrating α-keto-amide calpain inhibitors as potential treatment for muscular dystrophy

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Abstract—Dipeptide-derived α-keto-amide compounds with potent calpain inhibitory activity have been identified. These reversible covalent inhibitors have IC_{50} values down to 25 nM and exhibit greatly improved activity in muscle cells compared to the reference compound MDL28170. Several novel calpain inhibitors have shown positive effects on histological parameters in an animal model of Duchenne muscular dystrophy demonstrating their potential as a treatment option for this fatal disease.

Calpains are calcium-activated neutral proteases belonging to the papain superfamily of cysteine proteases; the best characterized are calpains I and II. Calpains are widely distributed in mammalian cells and have been implicated in a variety of diseases such as stroke, Alzheimer's disease, spinal cord injury, cardiac ischemia, muscular dystrophy, and cataract.[1] Thus, in recent years, calpain inhibition has become an important pharmacological strategy to develop novel therapies.

Our drug discovery program for novel calpain inhibitors emerged from our interest in finding a treatment for Duchenne muscular dystrophy (DMD).[2] Muscular dystrophies are a group of neuromuscular diseases characterized by progressive weakness and degeneration of body musculature. The most prevalent form is DMD, an X-chromosome-linked inherited disease with an incidence rate of 1 in 3500 newborn boys worldwide affecting about 40,000 male patients in Europe and North America. This fatal disease manifests between the age of two and six, confines affected teenage children to wheelchairs, and ultimately leads to death at early age. Treatment options are at present very limited and include artificial respiration, orthopedic surgery, and the short-term use of glucocorticoids primarily to limit the disease-associated inflammation.

A pharmacological approach using enzyme inhibitors is currently regarded as a possible therapeutic strategy for the treatment of DMD. Absence of functional dystrophin protein in DMD muscle leads to impaired muscle cell membrane integrity during cycles of contraction and relaxation. Calcium influx through membrane lesions causes abnormal calpain activation and proteolysis and, therefore, contributes to muscle cell deterioration. Subsequent protein degradation by the proteasome pathway further promotes muscle fiber damage and muscle weakness. Therefore, activation of the cytosolic Ca^{2+}-dependent cysteine proteases calpain I and II is believed to be a critical step in the pathogenesis of DMD. Consequently, it is considered that inhibition of calpains holds the potential to slow down or stop disease progression by preventing muscle degeneration and necrosis.

A number of calpain inhibitors have been reported in the literature. Most of these are modified peptides containing reactive functional groups that interact with the active-site cysteine thiol of calpain. These compounds can be classified as either irreversible or reversible inhibitors. Irreversible inhibitors include peptidyl

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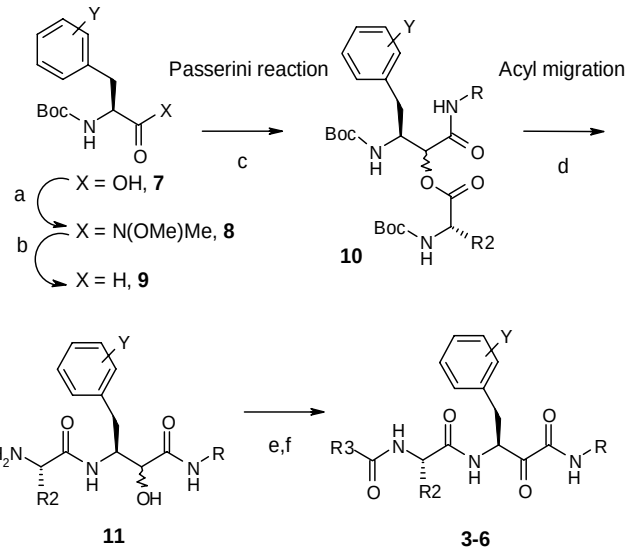
halo-ketones,[3] diazo-ketones,[4] epoxy-succinyls,[5] and other derivatives. They contain reactive functional groups which can be nucleophilically displaced by the cysteine thiol to form a sulfide, thereby permanently inactivating calpain. Reversible inhibitors include peptidyl aldehydes[6] like the natural product leupeptin **1** (Fig. 1) and activated ketones[7] like α-keto-esters, α-keto-acids, and α-keto-amides. They inactivate the enzyme in a transient manner by forming a reversible covalent bond (hemithioacetal or -ketal) with the cysteine thiol. However, most of these inhibitors have unsatisfactory pharmacological properties regarding selectivity, solubility, stability, or cellular penetration.[1b]

Starting from known calpain inhibitor lead compounds such as MDL28170 **2** (Fig. 1),[8] we initiated a lead optimization program to develop reversible calpain inhibitors **3**–**6** which possess a chemically and metabolically stable warhead and which show improved uptake into muscle cells.[9] We have focused on the synthesis and evaluation of dipeptide-derived α-keto-carbonyl compounds.

Depending on the structure of the target compounds and the availability of the starting materials, two synthetic routes were used. The first method (method A) involved the Passerini reaction as the key step to generate the precursor **11** of the required keto-amide functionality.[10] As displayed in Scheme 1, aldehydes **9** were synthesized from acids **7** under known conditions[7c] and reacted with isocyanides and Boc-protected amino-acids using Passerini conditions to yield **10**. α-Acyloxyamides **10** underwent an acyl migration after Boc removal and treatment with triethylamine to give the α-hydroxy-amides **11**. Subsequent coupling with different acids under standard conditions (HBTU or EDCI/HOBt) followed by Dess–Martin oxidation provided the α-keto-amides **3**–**6**.



Scheme 1. Reagents and conditions: (a) NHMe(OMe), EDCI, NMM, CH_2Cl_2, 0 °C to rt (~90%); (b) $LiAlH_4$, Et_2O, 0 °C (60–95%); (c) RNC, Boc–AA_2–CO_2H, CH_2Cl_2, rt; (d) 1—TFA/CH_2Cl_2 (1/3), 0 °C to rt; 2—NEt_3, CH_2Cl_2, rt (40–55%, three steps); (e) R_3–COOH, HBTU, DIPEA, DMF, 0 °C to rt (80–95%); (f) Dess–Martin periodinane, DMSO, CH_2Cl_2, 0 °C to rt, (25–85%).

The alternative protocol (method B) utilized the Wasserman acyl cyanophosphorane oxidative cleavage (Scheme 2).[11] Coupling of the acids **7** with cyanomethyl-triphenylphosphonium ylide afforded phosphoranes **12**. Subsequent ozonolysis of the phosphorus–carbon double bond at low temperature resulted in the generation of reactive acyl nitrile that was then displaced by the appropriate nucleophiles to yield the α-keto-carbonyl intermediates **13**. Deprotection in acidic medium and coupling of the left moieties provided the final compounds **3**–**6** as a 1:1 mixture of diasteromers at P1.

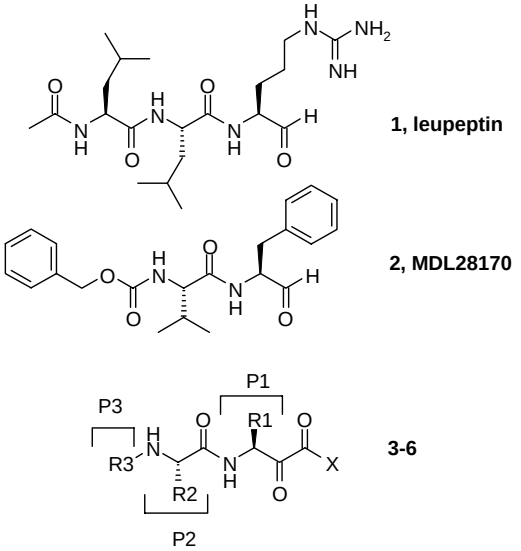
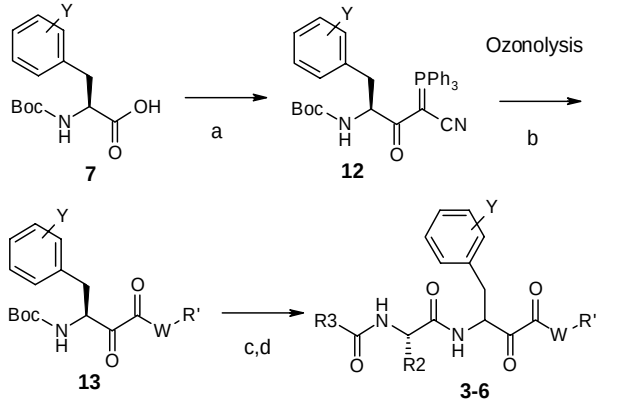


Figure 1. Leupeptin **1**, MDL28170 **2**, and general structure of the dipeptide-derived α-keto-carbonyl calpain inhibitors **3**–**6**.



Scheme 2. Reagents and conditions: (a) $Ph_3P{=}CHCN$, EDCI, DMAP, CH_2Cl_2, 0 °C to rt, 14 h (48–83%); (b) 1—O_3, CH_2Cl_2, −78 °C, 45 min; 2—Ar flushing, −78 °C, 5 min; 3—+R′WH, −78 °C for 1 h, then to rt, 14 h (esters: 24–45%; amides: 40–85%); (c) HCl (3 M) in dioxane, 0 °C, 3 h (esters: 35–65%; amides: quant.); (d) R_3–COOH, EDCI, HOBt, NMM, CH_2Cl_2, 0 °C to rt, 14 h (esters: 25–45%; amides: 55–85%).

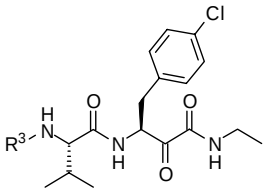
According to numerous publications, the P1 and P2 residues are quite well defined to achieve the optimal in vitro activity: preferred P1 residues are large hydrophobic groups like phenylalanine and preferred P2 residues are valine and leucine.[1] Therefore, we decided to focus first on the P3 moiety (Fig. 1) by replacing the benzyloxycarbonyl group (Cbz) of MDL28170 with a residue which would improve the uptake of the compounds into muscle cells. Calpain I inhibition was initially determined in a cell-free assay by measuring the time course of the hydrolysis of a fluorogenic substrate Suc-Leu-Tyr-AMC by purified porcine calpain I (increase in fluorescence over 30 min, λ_{ex} = 360 nm, λ_{em} = 440 nm).[12] Subsequently, the inhibitory activity within muscle cells was determined in a cell-based assay using cultured C2C12 myoblasts which were loaded with the fluorogenic substrate Suc-Leu-Tyr-AMC prior to the experiment. Cellular calpains were activated by incubation with calcium ionophore 4-Br-A23187 (10 μM) in the presence of increasing concentrations of the inhibitor.

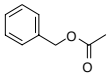
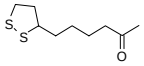
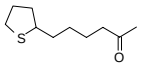
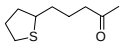
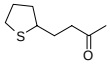
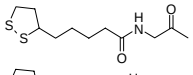
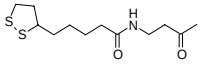
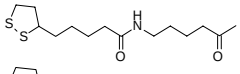
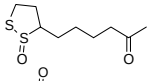
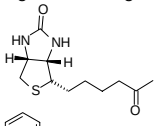
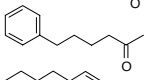
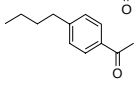
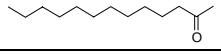
Using the Passerini strategy, we prepared a series of inhibitors with ethyl α-keto-amide as warhead to circumvent the chemical and metabolic instability of the aldehyde. We investigated different residues known to be carried by biological transporters into the human cells. A non-polar lipophilic residue, lipoyl **3b**, was identified to mediate uptake into muscle cells as measured by improved intracellular activity of the compound. Next, we evaluated the influence of small changes on this type of moiety as summarized in Table 1 with focus on a series **3a**–**m** characterized by valine in P2 and *p*-Cl-phenylalanine in P1, respectively.

Removal of one sulfur atom resulted in another potent inhibitor **3c** with a significantly improved intracellular activity compared to the compound **3a** bearing a Cbz group. Interestingly, shortening of the chain length by one (**3d**) or two carbons (**3e**) led to a loss of potency in the cellular assay (5–8-fold decrease) while keeping the activity against calpain I in the cell-free assay. Addition of a spacer between the lipoyl residue and the P2 moiety (**3f**–**h**) also resulted in a reduced intracellular potency while the inhibitory activity as measured in a cell-free assay remained in the same range. More polar analogs like sulfoxide **3i** and biotin **3j** derivatives were investigated as well. The sulfoxide derivatives were isolated in some cases as a side product of the final oxidation step. These compounds usually kept the inhibitory potency against the enzyme but exhibited poor intracellular activity (14–17-fold decrease). Replacement of the sulfur ring by a phenyl (**3k**) or attachment of more lipophilic residues like 4-*n*-butylbenzoyl (**3l**) or lauroyl (**3m**) resulted in weaker cell permeability or overall potency, once again demonstrating the superiority of the lipoyl residue over other lipophilic moieties.

Having identified the lipoyl group as the most potent P3 residue for improving the permeability into the muscle cells, a study to confirm the best P2 residues was undertaken (Table 2). With respect to the P2 position, our results generally are in agreement with previous observations:[1] the S2 pocket of calpain is quite tight and can tolerate only small lipophilic residues. Leucine and valine are the preferred residues as illustrated in **3b** and **4d** which are the most potent compounds in both enzymatic and cellular assays. Surprisingly, *tert*-butylglycine, reported to be a potent P2 moiety,[6b] resulted in the inactive compound **4b**. Large groups like cyclohexyl (**4c**) proved to be detrimental. In another series, replacement of leucine by its polar isoster threonine led to poor

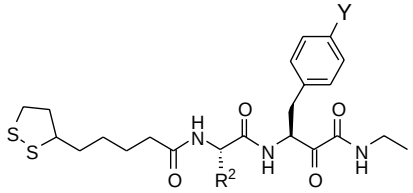
Table 1. Inhibition of calpain I by P3 analogs **3a**–**m**



Compound	R^3	Calpain I inhibition cell-free[b] IC_{50} [μM][a]	Calpain I inhibition myoblast[c] IC_{50} [μM][a]
1	Leupeptin	0.08	0 % at 10 μM
2	MDL28170	0.02	10
3a		0.09	15
3b		0.06	0.35
3c		0.10	0.40
3d		0.12	2.0
3e		0.10	3.2
3f		0.03	1.0
3g		0.04	30
3h		0.10	20
3i		0.08	6.0
3j		0.10	5.0
3k		0.06	1.5
3l		0.15	25
3m		>1.0	n.d.

[a] Values are means of triplicates (n.d. = not determined).
[b] Hydrolysis of fluorogenic substrate Suc-Leu-Tyr-AMC by purified porcine calpain I.
[c] Activation of cellular calpain with Ca^{2+}-ionophore Br-A-23187 and hydrolysis of fluorogenic substrate Suc-Leu-Tyr-AMC by cellular calpain.

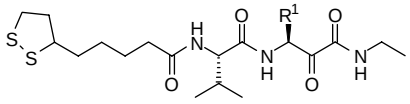
Table 2. Inhibition of calpain I by P2 analogs **4a–e**



Compound	R^2	Y	Calpain I inhibition, cell-free IC_{50} [μM][a]	Calpain I inhibition, myoblast IC_{50} [μM][a]
3b		Cl	0.06	0.35
4a		Br	0.18	9.0
4b		Cl	>1.0	n.d.
4c		Br	>1.0	n.d.
4d		Cl	0.02	0.39
4e		Cl	0.10	3.5

[a] Values are means of triplicates (n.d. = not determined).

Table 3. Inhibition of calpain I by P1 analogs **5a–s**



Compound	R^1	Calpain I inhibition, cell-free IC_{50} [μM][a]	Calpain I inhibition, myoblast IC_{50} [μM][a]
3b	4-Cl-$PhCH_2$–	0.06	0.35
5a	$PhCH_2$–	0.08	3.5
5b	4-F-$PhCH_2$–	0.10	2.5
5c	4-Br-$PhCH_2$–	0.06	0.20
5d	4-MeO-$PhCH_2$–	0.12	0.22
5e	4-Me-$PhCH_2$–	0.07	0.35
5f	4-CF_3-$PhCH_2$–	0.20	1.1
5g	4-*t*-Bu-$PhCH_2$–	0.40	1.8
5h	4-CN-$PhCH_2$–	0.12	5.0
5i	4-OH-$PhCH_2$–	0.15	2.5
5j	3-CF_3-$PhCH_2$–	0.10	15
5k	2,4-Di-Cl-$PhCH_2$–	0.10	40
5l	3,4-Di-Cl-$PhCH_2$–	0.08	10
5m	3,4-Di-F-$PhCH_2$–	0.10	0.70
5n	2-Thienyl-CH_2–	0.10	6.0
5o	*c*-Hexyl-CH_2–	0.25	3.0
5p	*i*-Bu–	0.50	0.25
5q	Ph–	0.40	2.0
5r	Ph-CH_2-CH_2–	>1.0	>10
5s	$(Ph)_2CH$–	>1.0	n.d.

[a] Values are means of triplicates (n.d. = not determined).

inhibitors as well (data not shown) providing evidence that the S2 pocket cannot accommodate polar groups.

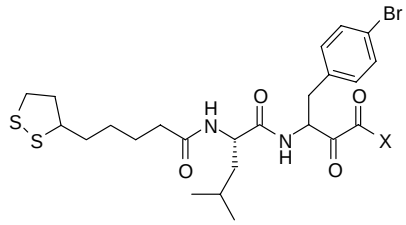
As illustrated in Table 3, we also initiated a SAR study to identify more potent P1 residues. We found that the use of substituted phenylalanines as the P1 moiety afforded inhibitors with a better binding to the enzyme usually with IC_{50} values below 0.15 μM. Calpain can accommodate different substitution patterns on the phenyl ring but the position of the substituent has a significant influence on the intracellular activity. Substitution at the 4-position with hydrophobic groups provided compounds with improved uptake into the muscle cells (up to 17-fold improvement compared to the unsubstituted analog **5a**). Chloro- (**3b**), bromo- (**5c**), methoxy- (**5d**), and methyl- (**5e**) were among the best substituents. Di-substitution (**5k–m**) as well as the use of polar substituents (**5i**) was detrimental to the cellular activity. Replacement of the phenyl ring by a thienyl (**5n**) or a cyclohexyl ring (**5o**) or by an isopropyl group (**5p**) did not improve inhibitory activity. Interestingly, the use of phenylglycine (**5q**) maintained moderate activity, whereas increase of the chain length by one carbon resulted in a loss of activity (**5r**). Bulky α-branched phenylalanines led to inactive compounds like **5s**.

We finally evaluated the effect of changes in the warhead group on inhibitory potency as summarized in Table 4. In the lipoyl-Leu-4-BrPhe series, changing the ethyl α-keto-amide (**6a**) to the α-keto-acid (**6b**) resulted in a similar potency against calpain but in a diminution of the intracellular activity likely due to its polarity preventing a good membrane permeability. The corresponding ethyl α-keto-ester **6c** proved to be inactive against the enzyme (IC_{50} > 10 μM). Therefore, we decided to focus further efforts on the α-keto-amide series. *N*,*N*-disubstituted α-keto-amide analogs as exemplified with **6d** were much less potent than *N*-monosubstituted α-keto-amides. This observation is consistent with previous work[7b] and could indicate that the NH of the α-keto-amide functional group forms a hydrogen bond with an amino-acid residue of the active-site of calpain. Primary amide (**6e**) and other *N*-alkyl or *N*-alkylaryl amide derivatives were also examined (data not shown). They usually exhibited similar potency as **6a**, but the α,α-disubstituted *N*-alkyl residues like **6g** were detrimental for the binding to the enzyme. In order to prepare more water soluble compounds, polar moieties were also evaluated. Intracellular potency of weakly basic residues on the alkyl chain like pyridyl (**6h**) or morpholinyl (**6i**) was comparable to the one of non-polar moieties, whereas strongly basic substituents like piperidine led to a significant decrease of the intracellular activity with IC_{50} values above 5 μM (data not shown).

As depicted in Table 5, biochemical profiling against other biologically relevant proteases demonstrated good selectivity versus caspase 3 and thrombin. In the case of cathepsin B, limited selectivity was observed. Some compounds (**5c**) proved to be moderate 20S-proteasome inhibitors. This property could be beneficial since both calpain- and proteasome-mediated proteolytic pathways are involved in muscular dystrophies.

Finally, we examined the influence of the chiral center of the lipoyl residue by synthesizing the two isomers **6a**-(*R*) and **6a**-(*S*) starting from (*R*)-lipoic acid and (*S*)-lipoic

Table 4. Inhibition of calpain I by P′ analogs **6a–i**



Compound	X	Method	Calpain I inhibition cell-free IC_{50} [μM][a]	Calpain I inhibition myoblast IC_{50} [μM][a]
6a	–NHEt	A	0.02	0.5
6b	–OH	B	0.05	5.0
6c	–OEt	B	>10	4.5
6d	$-N(CH_2CH_2)_2$-O	B	>1.0	n.d.
6e	$-NH_2$	B	0.02	0.25
6f	–NH-*c*-Hex	B	0.07	0.28
6g	–NH-*t*-Bu	A	>1.0	n.d.
6h	$-NH(CH_2)_2$-2-pyridyl	B	0.23	0.8
6i	$-NH(CH_2)_3$-morpholinyl	B	0.12	1.0

[a] Values are means of triplicates (n.d. = not determined).

Table 5. Inhibitory activity profiling

Compound	CalpI IC_{50} [μM][a]	CathB IC_{50} [μM][a]	20S-Proteasome IC_{50} [μM][a]	Casp3 % inhib. at 10 μM[a]	Thrombin % inhib.at 10 μM[a]
2	0.02	0.10	>1.0	0	0
3b	0.06	0.10	0.50	17	36
4d	0.02	0.12	0.52	0	20
5c	0.06	0.04	0.18	0	0
6a	0.02	0.65	0.62	0	30

[a] Values are means of triplicates.

acid.[13] The inhibitory activity of **6a**-(*R*) and **6a**-(*S*) was similar to that of the racemate **6a** with IC_{50} value of 0.039 and 0.024 μM, respectively, in the cell-free assay and IC_{50} value of 0.4 μM for both isomers in the myoblast assay. Therefore, compounds were used as racemates in the animal model.

The best inhibitors were tested in vivo in *mdx* mice, a well-established animal model for DMD (ip treatment, every second day between the third and seventh week of age, 20 mg/kg, PEG200/saline (1:1) vehicle). A well-documented hallmark of dystrophic muscles seen in *mdx* mice is the increased variability of muscle fiber size diameters indicating elevated muscle fiber turnover. This variability in muscle fiber diameters was quantified by calculating the variance coefficient of the minimal Feret's diameter of all muscle fibers in a given muscle.[14] Several inhibitors showed a normalized histological appearance, as illustrated in Figure 2, and improved variance coefficient of the muscle fiber diameters. As summarized in Table 6, between 25% and 30% improvement was observed in diaphragm muscle upon application of the keto-amides **3b**, **5c**, and **6a**. Similar histopathological recovery was achieved by overexpression of calpastatin (a specific endogenous calpain inhibitor) in *mdx* mice.[15]

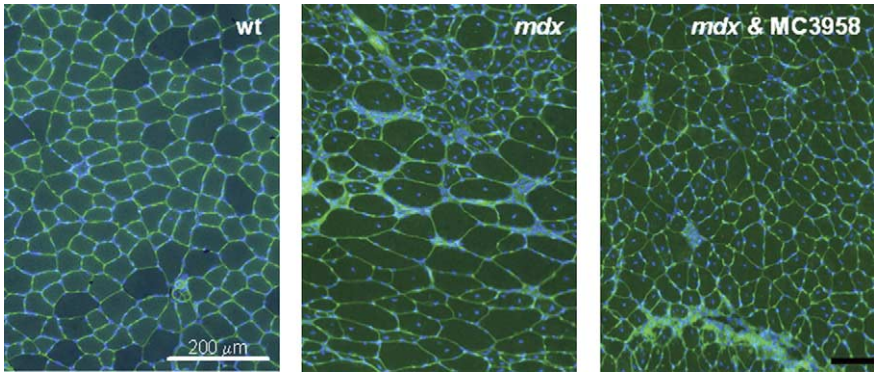
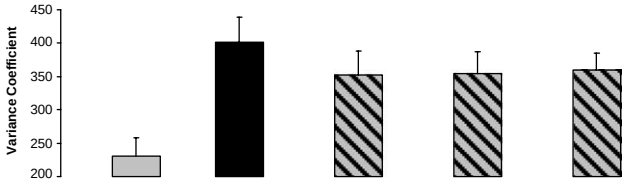


Figure 2. Representative images showing the normalization of the fiber size distribution in diaphragm muscle of *mdx* mice upon four weeks application of **6a**.

Table 6. Variance coefficient of muscle fiber size in diaphragm muscle



	Wild type	*mdx*	**3b**[c]	**5c**[c]	**6a**[c]
Mean[a]	231.2	401.6	351.5	354.7	359.7
% Red.[b]			29.4	27.5	24.6
Mice	12	27	5	5	13
p-value[d]		< 0.001[e]	0.013[f]	0.008[f]	0.001[f]

[a] Variance coefficient.[14]
[b] % Reduction in the variance coefficient of muscle fiber sizes as compared to untreated mice (= 100%).
[c] *mdx* treated with compound.
[d] *p*-value unpaired Student's *t*-test.
[e] Comparison against wild type.
[f] Comparison against untreated *mdx*.

We have described the preparation of novel dipeptide-derived α-keto-amides as potent calpain inhibitors. These compounds possess a lipoyl moiety at the P3 position and demonstrate an improved calpain inhibitory activity in cultured muscle cells compared to the reference compound MDL28170. Additionally, some derivatives displayed moderate 20S-proteasome inhibition which could be advantageous since both calpain- and proteasome-mediated proteolytic pathways are involved in DMD. In the *mdx* mouse model, several compounds significantly improved relevant histopathological parameters demonstrating their potential as a treatment for this devastating disease.

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