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Exploration of Peptidyl Hydrazones as Water-Soluble Calpain Inhibitors

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Abstract—A series of peptidyl hydrazones was synthesized, and their inhibitory activity against μ-calpain and water-solubility were measured. Among these compounds, *N,N*-dimethyl glycyl hydrazone **6**, which inhibited μ-calpain with IC_{50} of 0.37 μM, possessed the appropriate water-solubility. Furthermore, hydrazone **6** was found to possess the excellent in vitro metabolic stability.

Introduction

Calpains are a family of calcium dependent neutral cysteine proteases comprised of ubiquitous calpains such as μ- and *m*-calpain, and tissue-specific calpains such as p94 (muscle), Lp82 (lens), Lp85 (lens), nCl-2 and -2′ (stomach) and Rt88 (retina).[1] These enzymes play important roles in various biological processes and many diseases such as central nervous system (CNS) disease, Alzheimer's disease, muscular dystrophy and cataract.[2] Therefore, much attention has been paid to the rational design and synthesis of calpain inhibitors.[3] Peptidyl aldehyde[4] and α-ketoamide[5] are reversible calpain inhibitors, which form hemithioacetal or ketal with the active SH of cysteine residue of the enzymes. Epoxysuccinyl derivative,[6] peptidyl halomethyl ketone and (acyloxy)methyl ketone[7] irreversibly inhibit calpains via alkylation of the active center SH. Several nonpeptidic calpain inhibitors also have been described.[8] Among the compounds, peptidyl aldehyde, SJA6017 (**1**) and α-ketoamide, AK-295, have demonstrated in vivo efficacies.[9]

Peptide-like drugs sometimes have problems such as water-insolubility, metabolic instability, absorption and the availability for both oral and parenteral dosage forms.[10] Even if the compound makes high affinity binding to the target protein in vitro, that efficacy is not so high because of poor absorption and pharmacokinetics. It is one of the main reasons for attrition in the drug development process.[11] Recently, many medicinal chemists are conscious of these problems, but there are still many unsolved problems.

In our drug developments, peptidyl hydrazones were selected as new template for calpain inhibitor as shown in Figure 1. We tried to enhance the water-solubility of the inhibitor, by bearing various hydrazines at R^1. Now, we report here the synthesis, inhibitory activity, water-solubility and metabolic stability of peptidyl hydrazones as μ-calpain inhibitors.

Chemistry

The synthesis of peptidyl hydrazones was accomplished by the condensation of the appropriate peptidyl aldehydes with hydrazine. A representative example is shown in Scheme 1. The peptidyl aldehyde **1**, which was prepared by a general peptide coupling method from 4-fluorophenylsulfony chloride, L-valine and L-leucinol as materials in several steps, followed by a DMSO oxidation using sulfurtrioxide pyridine complex, as reported previously.[12] Reaction of aldehyde **1** with semicarbazide

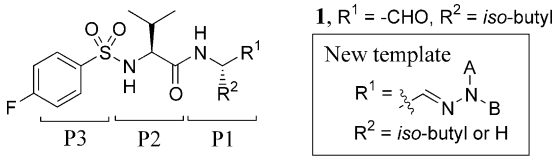

Figure 1. Strategy for new calpain inhibitor.

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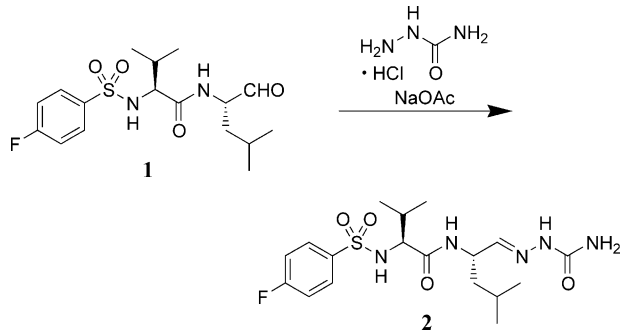


Scheme 1.

hydrochloride in the presence of sodium acetate provided peptidyl hydrazone **2**.[13] Compounds **3**–**7** (Table 1) were prepared by using a similar method.

Protease assay and water-solubility

The protease assay of the peptidyl hydrazones was carried out according to a published method[14] by using porcine erythrocyte μ-calpain (nacalai tesque). The water-solubility was determined by shaking the mixture of the sample in buffer (pH 4–7) for 5 h at 25 °C. The suspension was filtered (0.45 μm) and the filtrate was analyzed for drug content by HPLC.[15]

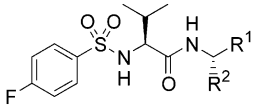
Metabolic stability

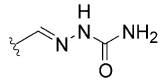
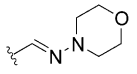
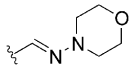
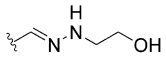
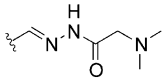
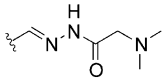
Metabolic stability of peptidyl hydrazone **6** and aldehyde **1** in the presence of human liver S9 was examined by measuring the loss of the drug as follows. A mixture containing the test compound (2 μg/mL), β-NAD^+ (0.8 mM), β-$NADP^+$ (0.8 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (10 U/mL), $MgCl_2$ (30 mM), human liver S9 (1 mg protein/mL, Gentest Corp.) and Tris–HCl (80 mM, pH 7.4), was incubated at 37 °C for 0, 15, 30 and 60 min. The reaction was quenched with methanol and centrifuged. The supernatant was concentrated and the sample was analyzed for drug content by HPLC.[15]

Results and Discussion

Table 1 shows inhibitory activity (IC_{50}) against μ-calpain and water-solubility of the synthesized compounds. Hydazones containing *iso*-butyl group at R^2, **2**, **3**, **5** and **6**, are potent μ-calpain inhibitors which show IC_{50} values of 10^{-7} M, although these compounds are about 10-fold less potent than parent aldehyde **1**. The most potent compound is hydroxyethyl hydrazone **5** that inhibited μ-calpain with IC_{50} of 0.19 μM. To study the importance of P1 residue, **4** and **7** were synthesized and

Table 1. Inhibitory activity (IC_{50}) against μ-calpain and water-solubility



Compd	R^1	R^2	IC_{50} (μM) μ-calpain	Water-solubility (mg/mL)			
				pH 4	pH 5	pH 6	pH 7
2		*iso*-Butyl	0.68	0.433	nd[a]	nd	nd
3		*iso*-Butyl	0.79	0.0673	0.0633	0.0608	0.0582
4		H	6.2	1.26	0.840	0.692	0.667
5		*iso*-Butyl	0.19	0.228	0.234	0.226	0.293
6		*iso*-Butyl	0.37	4.33	1.98	0.702	0.213
7		H	>10	>5	>5	>5	>5
1		*iso*-Butyl	0.035	0.102	nd	0.100	0.105

[a]nd, not determined.

tested. Removal of the *iso*-butyl group decreased the inhibitory activity (**3** vs **4** and **6** vs **7**). Thus, it was considered that the *iso*-butyl group at P1 position was important due to the hydrophobic interaction with μ-calpain. This SAR is consistent with that of other type of inhibitors such as peptidyl aldehyde and α-ketoamide.[16]

Hydroxyethyl hydrazone **5** was synthesized with a purpose of increasing the water-solubility due to increasing the hydrophilicity with hydroxy group. On the similar reason, hydrazone **3** was synthesized, because morpholine moiety was often introduced in the pharmacophore to increase the water-solubility.[17] However, the both of the compounds were practically insoluble in the buffer solutions (pH 4–7). Morpholine hydrazone **4** without *iso*-butyl group at R^2 exhibited an increase of water-solubility compared to the morpholine hydrazone **3** with *iso*-butyl group at R^2, whereas hydrazone **4** was a weak inhibitor. More importantly, *N,N*-dimethyl glycyl hydrazone **6** had a good water-solubility (4.33 and 1.98 mg/mL in pH 4 and pH 5 buffer solutions respectively) while maintaining a potent activity. As for the *N,N*-dimethyl glycyl hydrazones as well as morpholine compounds, removal of *iso*-butyl group increased the water-solubility (**6** vs **7**). Therefore, *iso*-butyl group is indispensable to maintain the inhibitory activity in hydrazone series of calpain inhibitors, although it obstructs to increase the water-solubility.

Since hydrazone **6** exhibited the adequate inhibitory activity and water-solubility in pH range of medical use, we studied the in vitro metabolic stability of hydrazone **6** and aldehyde **1** in the presence of human liver S9. As shown in Figure 2, the remaining of hydrazone **6** was higher than that of parent aldehyde **1** in this experiment. Thus, conversion of the active aldehyde into *N,N*-dimethyl glycyl hydrazone remarkably improved the in vitro metabolic stability.

In conclusion, although *N,N*-dimethyl glycyl hydrazone **6** is a 10 times less potent than aldehyde type calpain inhibitor **1**, it possesses appropriate water-solubility and excellent in vitro metabolic stability. Therefore, hydrazone **6** is very promising candidate for the further development since it is possible to exhibit improved pharmacokinetic properties. In vivo pharmacokinetic and pharmacodynamic studies will be conducted and will be discussed in somewhere soon.

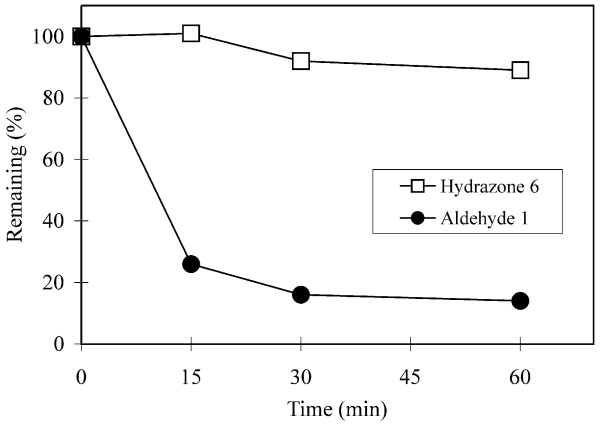


Figure 2. Metabolic stability of peptidyl hydrazone **6** and aldehyde **1** in the presence of human liver S9.

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13. Hydrazone **2**: colorless crystals. Mp 100–102 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.70 (d, 3H, J = 8.4 Hz), 0.76–0.81 (m, 9H), 1.00–1.23 (m, 3H), 1.74–1.86 (m, 1H), 3.54 (m, 1H), 4.19 (m, 1H), 6.22 (s, 2H), 6.99 (d, 1H, J = 4.2 Hz), 7.32–7.38 (m, 2H), 7.79–7.83 (m, 2H), 7.90–7.93 (m, 2H), 9.85 (s, 1H). MALDITOF-MS $[M+H]^+$ calcd 430.192, found 430.186. Hydrazone **6**: colorless crystals. Mp 162–163 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.84 (d, 3H, J = 5.1 Hz), 0.85 (d, 3H, J = 6.9 Hz), 0.88 (d, 3H, J = 4.8 Hz), 0.96 (d, 3H, J = 6.9 Hz), 1.33–1.35 (m, 3H), 2.15 (m, 1H), 2.32 (s, 6H), 3.07 (s, 2H), 3.58 (m, 1H), 4.51 (m, 1H), 5.63 (m, 1H), 6.59 (d, 1H, J = 7.5 Hz),

7.11–7.18 (m, 2H), 7.60 (d, 1H, $J = 3.6$ Hz), 7.85–7.90 (m, 2H), 9.95 (s, 1H). Anal. calcd for $C_{21}H_{34}FN_5O_4S$: C, 53.49; H, 7.26; N, 14.85. Found: C, 53.20; H, 7.36; N, 14.61.

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15. Hydrazone: column; Shiseido Capcell PAK C18 UG120 (size 4.6×100 mm), mobile phase; CH_3CN/10 mM Tris–HCl buffer (pH 8) = 30:70, column temperature 40 °C, detection: 250 nm, flow rate: 1.0 mL/min. Aldehyde **1**: column; YMC-Pack ODS A-303 (size 4.6×250 mm), mobile phase; CH_3CN/H_2O/TFA = 30:70:1, column temperature 45 °C, detection: 250 nm, flow rate: 1.0 mL/min.

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