Brief Articles

Structure−Activity Relationship Study and Drug Profile of *N*-(4-Fluorophenylsulfonyl)-L-valyl-L-leucinal (SJA6017) as a Potent Calpain Inhibitor

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Novel *N*-arylsulfonyldipeptidyl aldehyde derivatives were prepared by DMSO oxidation from the corresponding dipeptide alcohol, and their potencies as calpain inhibitors were evaluated in vitro. Among them, *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal (**8**, SJA6017) potently inhibited calpains. **8** also inhibited cathepsin B and L but did not inhibit other cysteine proteases (interleukin 1β-converting enzyme), serine proteases (trypsin, chymotrypsin, thrombin, factor VIIa, factor Xa), or proteasome. Preliminary cytotoxicity studies of **8** exhibited a relatively safe profile.

Introduction

The calpains (EC 3.4.22.17), which belong to the cysteine protease family, exist ubiquitously in cytosol and are activated by calcium. There are 16 kinds of calpain identified in mammalian tissue which can be divided into two groups—calpains that are tissue-specific and calpains that are ubiquitously expressed.[1] Among them, two representative types of calpain isoform have been well studied, μ-calpain (or calpain I) and m-calpain (or calpain II), which differ in their in vitro requirements for calcium. The distribution and biochemical property of calpains have been the subject of several reviews.[2] Overactivation of calpain has also been implicated in a variety of disorders such as muscular dystrophy, Alzheimer's disease, stroke, ischemic disease, and cataract.[3] As one of the first potent peptidyl aldehyde cysteine protease inhibitors, leupeptin was found in culture filtrates of various species of actinomycetes.[4] Unfortunately, this compound showed poor cell penetration and lacked enzyme specificity. Chemical modification studies of leupeptin as the lead compound show that peptidyl aldehyde type calpain inhibitors, calpeptin or MDL28170, were potent cell penetrative compounds.[5] It has been known that calpains favorably cleaved the C-terminal side (P_1 position) of tyrosine, methionine, or arginine next to leucine or valine (P_2 position).[6] And additionally, Harris[7] reported *N*-benzyloxycarbonyl-leucyl-tyrosinal and *N*-benzyloxycarbonyl-valyl-tyrosinal were potent calpain inhibitors, and Iqbal[8] also reported *N*-benzyloxycarbonyl-leucyl-valinal, *N*-benzyloxycarbonyl-*tert*-butylglycyl-leucinal, and *N*-benzyloxycarbonyl-leucyl-(O-benzyl)tyrosinal were the most potent calpain inhibitors.

In this article, we describe the substituent effect of *N*-arylsulfonyldipeptidyl aldehydes on calpain inhibition and the safety, permeability, and metabolic profiles of *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal **(8)**, the most potent inhibitor.

Results and Discussion

Chemistry. The series of *N*-sulfonyldipeptide aldehyde was prepared as outlined in Scheme 1. Amino acids **1** were coupled with arylsulfonyl chloride by the Shotten−Baumann method to generate the sulfonylated amino acids **2**. Dipeptidyl alcohols **4** were synthesized by the active ester **3** prepared from **2** and various amino alcohols. A DMSO oxidation[9] of compound **4** using sulfur trioxide−pyridine complex with triethylamine gave dipeptidyl aldehydes **(5)**.

Structure−Activity Relationship. Inhibitory activity of synthesized compounds was evaluated by the reported method[10] summarized in Table 1. When the less bulky group (Me) was incorporated in the P_3 position (**17**), calpain inhibitory activity decreased compared to the aromatic ring, and when alanine was incorporated into the P_1 position (**11**), the activity decreased. These results suggest that an aromatic group at the P_3 position and a certain size of bulky amino acid residual aldehyde at the P_1 position are required for potent calpain inhibition. When the P_2 position was changed from valine to norvaline (**18**) or norleucine (**19**), the activity also decreased. A long chain was not acceptable at the P_2 position. Many studies have been done to identify the preferences at the P_1 and P_2 position of a peptidyl inhibitor. Harris revealed that a certain size of sterically hydrophobic amino acid at P_1 or P_2 position was important.[7] Iqbal mentioned that Leu and Phe were preferred at P_2/P_1 for calpain.[8] Our results basically agreed with the previous results. Also, P_2 N−H was required, probably bound to calpain with hydrogen

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Scheme 1. A General Synthetic Procedure for *N*-Arylsulfonyldipeptide Aldehyde

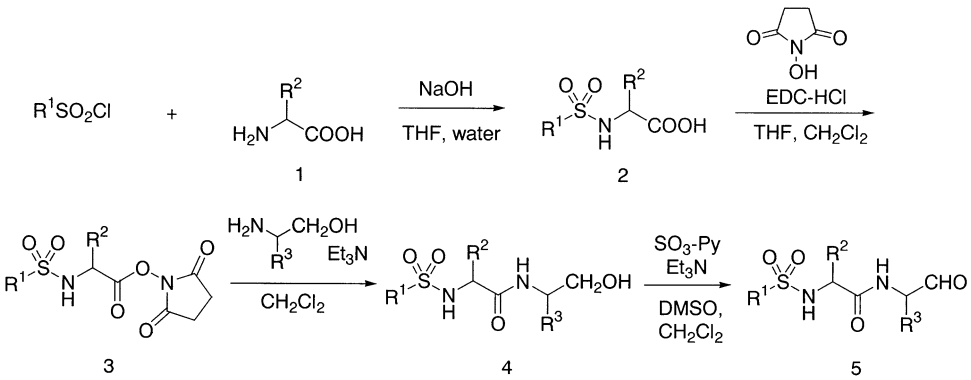
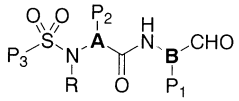


Table 1. Variation of the P_1 and P_3 Substituent on *N*-sulfony-L-valylpeptidyl Aldehydes and Their Inhibitory Activities against μ-Calpain



compd	P_3	R	P_2	P_1	configuration A	configuration B	IC_{50} (nM) μ-calpain
6	2-Nap	H	L-Val	L-Leu-H	L	L	10
7	2-Nap	H	L-Val	L-Phe-H	L	L	14
8	4-F-Ph	H	L-Val	L-Leu-H	L	L	7.5
9	4-F-Ph	H	L-Val	L-Phe-H	L	L	27
10	4-F-Ph	H	L-Val	L-Trp-H	L	L	23
11	4-F-Ph	H	L-Val	L-Ala-H	L	L	630
12	4-Cl-Ph	H	L-Val	L-Leu-H	L	L	31
13	4-Cl-Ph	H	L-Val	L-Phe-H	L	L	14
14	4-Cl-Ph	H	L-Val	L-Trp-H	L	L	13
15	4-Me-Ph	H	L-Val	L-Leu-H	L	L	28
16	4-Me-Ph	H	L-Val	L-Phe-H	L	L	18
17	Me	H	L-Val	L-Leu-H	L	L	830
18	4-F-Ph	H	L-Nval	L-Leu-H	L	L	130
19	4-F-Ph	H	L-Nle	L-Leu-H	L	L	260
20	4-F-Ph	CH_3	L-Val	L-Leu-H	L	L	21000
21	4-F-Ph	H	L-Val	L-Leu-H	L	D	14000
22	4-F-Ph	H	L-Val	L-Leu-H	D	L	42000
23	4-F-Ph	H	L-Val	L-Leu-H	D	D	1000000

bonding since *N*-methylation dramatically decreased the inhibitory activity (**20** vs **8**). The importance of P_2 N−H has been reported by Iqbal.[8] Dolle also mentioned the importance of P_2 N−H on not only calpain but on other members of the papain superfamily and interleukin-1β converting enzyme.[11] Chatterjee, however, reported that the NHCO moiety at the P_2−P_3 region of peptide inhibitor is not a strict requirement for enzyme recognition.[12] Diastereomers and the enantiomer of **8** were distinctly weak inhibitors in comparison with **8** (vs **21**, **22**, **23**). It is important to keep the absolute configuration of each amino acid residue the same as the natural type amino acid for calpain inhibition. Yasuma[13] also reported the importance of *S*-configuration on cathepsin L inhibition. Chatterjee, however, revealed the importance of a D-configuration, which is unnatural amino acid configuration, at P_2 in their series of methansulfonydipeptidyl aldehydes.[14] To clarify the reasons for these two significant differences, further investigations are required such as computational docking study or crystallization of the enzyme−inhibitor complex.

Potency. Potency of **8** compared to known calpain inhibitors is shown (see detail in Supporting Information). The IC_{50}s of reference compounds reported here are approximately 5 times greater than literature IC_{50}s. One possible reason is that we use commercially available porcine enzyme instead of the human recombinant enzyme reported in the previous paper by Iqbal.[8] **8** was about 10 to 50 times more potent against μ-calpain, and 2 to 20 times more potent against m-calpain than the known calpain inhibitors. **8** was the most potent calpain inhibitor tested.

Enzyme Specificity. Compound **8** inhibited μ- (IC_{50} = 7.5 nM) and m-calpain (IC_{50} = 78 nM) and cathepsin B (IC_{50} = 15 nM) and L (IC_{50} = 1.6 nM) potently but did not inhibit other cysteine proteases (interleukin 1β-converting enzyme) (>100 μM), serine proteases (thrombin (>100 μM), factor VIIa (>100 μM), factor Xa (>60 μM), trypsin (>100 μM), chymotrypsin (>200 μM)), or proteasome (>200 μM). Generally speaking, peptidyl aldehyde inhibitors broadly inhibit both cysteine and serine proteases.[15,16] Recently, Wells found that *N*-(3,4-dihydro-6,7-ethylenedioxy-2-ethyl-2*H*-1,2-benzothiazine-3-carbonyl)-L-phenylalaninal 1,1-dioxide was a selective inhibitor of calpain I vs cathepsin B because of its rigid structure.[17] But, it is unclear whether inhibiting cathepsin B and L causes critical side effects for humans.

Toxicity. Cytotoxicity of **8**, **6**, and MDL28170 were evaluated by using human neuroblastoma SY5Y cells and rat cerebellar granule neurons. The 50% lethal concentrations (LD_{50}) of **8**, **6**, and MDL28170 were over 1000 μM, 9.8 μM, and 45.9 μM with SY5Y cells, respectively. **8** was approximately over 100 times less cytotoxic than **6** and 20 times less cytotoxic than MDL28170. The fact of **8** cell penetration was already reported with lens culture.[18,19] So **8** was relatively less cell toxic but could penetrate into cells. **8** exhibited no bacterial reverse mutation up to 5000 μg/plate from an Ames test result. Acute toxicity (rat, i.p. and p.o.) and repeat toxicity test (rat, p.o., for 2 weeks) of **8** were done. No death and no pathological damage were observed in all tests up to 100 mg/kg (i.p.) and 1000 mg/kg (p.o.).

Permeability with Caco-2 Cells. The apparent permeability of **8** was analyzed by a LC-MS/MS method, and P_{app} was calculated to be 1.41×10^{-6}, which suggests a relatively low permeability for this compound. However, only about 33% of dosed **8** was recovered, indicating that compound was bound to the cells or to the membrane. Even though the low recovery of **8** complicates interpretation of the experiment, the calculated P_{app} is likely indicative of low permeability for this compound.

Protein Binding to Mouse, Rat, Monkey, Dog, and Human Plasma. **8** was incubated with plasma

from mouse, rat, monkey, dog, and human, and the filtrate was analyzed. At 10 μM and 100 μM, respectively, **8** was 95.4% and 91.6% bound to human plasma, 89.9% and 90.2% bound to mouse plasma, 89.9% and 85.4% bound to rat plasma, 75.8% and 66.8% bound to dog plasma, and 95.1% and 90.5% bound to monkey plasma.

CYP450 Induction in Human Hepatocytes. Human hepatocytes were incubated with 1 and 10 μM of **8**. Incubation with **8** did not induce CYP450 1A2, 2C9, or 2C19 activity. However, **8** at 1 μM and at 10 μM increased 3A4 activity by 1.7 and 2.2-fold, respectively.

CYP450 Inhibition. Inhibition assays for cytochromes CYP450 1A2, 2C9, 2C19, 2D6, and 3A4 were performed separately. **8** does not inhibit CYP450 1A2 (IC_{50} > 1000 μM) but slightly inhibits 2C9 (IC_{50} = 492 μM), 2C19 (IC_{50} = 177 μM), 2D6 (IC_{50} = 291 μM), and 3A4 (IC_{50} = 81 μM with BFC as substrate, IC_{50} = 531 μM with BQ as substrate). However, **8** does not inhibit CYP450 1A2, 2C9, 2C19, 2D6, or 3A4 at concentrations less than 50 μM, which is generally considered to be the limit of physiologically relevant inhibition of CYP450 activity.

Comparison of Interspecies Metabolism in Human, Mouse, Dog, Monkey, and Rat Microsomes. The metabolic stability of **8** after incubation with microsomes in the presence and absence of NADPH was measured by LC-MS/MS. **8** was almost completely metabolized following incubation with human, mouse, monkey, and rat microsomes. After 60 min, only 2.5%, 2.1%, 0%, and 6.4% of parent compound remained with human, mouse, monkey, and rat microsomes, respectively. The control for monkey incubations with no NADPH also shown a loss of 82% of parent, suggesting that metabolism of **8** by monkey microsomes may be due to factors other than CYP450 activity. Interestingly, metabolism by dog microsomes appears to be less efficient with 68% of the parent compound remaining after 60 min. The structures of the metabolite have not been determined yet, but the appearance of diastreomers and enantiomers were determined in serum at PK/PD study.

Conclusion

We confirmed that an aromatic group at the P_3 position and a certain size of bulky amino acid residual aldehyde at the P_1 position is required for potent calpain inhibition in *N*-arylsulfonyldipeptidyl aldehydes. Our SAR study disagrees with previous studies on some points (P_2 N−H, stereo configuration). It is likely each inhibitor interacts with the enzyme by hydrogen bonding or hydrophobic interactions, and these differences are defined by the combination of P_1 and P_2 amino acid residues. It is difficult to determine which amino acids work best for P_1 and P_2 position, but the balance between them is important.

As the consequence, we found the best inhibitor against calpains was *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal (**8**) in this series of compounds based on potency and specificity. **8** was less toxic and had no activity on both P450 induction and inhibition, but Caco-2 permeability was poor and protein binding was relatively high. However, several previously reported in vivo studies regarding **8** have shown amelioration of cataract formation,[20] protection on ischemia-reperfusion injury in retina,[21] and protection on functional recovery after delayed administration in diffuse head injury.[22] Although **8** was the best candidate compound for clinically therapeutic use, there are problems remaining such as bioavailability or protein binding that need to be overcome by pharmaceutical formulation techniques or structural modifications.

Experimental Section

General Preparation for Sulfonyl Amino Acid (2): *N*-(4-Fluorophenylsulfonyl)-L-valine. To a solution of L-valine (11.7 g, 100 mmol) in 1 N NaOH (100 mL) and water (150 mL) were dropwise added 4-fluorobenzenesulfonyl chloride (17.5 g, 90 mmol) in THF (100 mL) and 1 N NaOH (100 mL) at the same time under an ice-cooled condition and stirred for 18 h at room temperature. The reaction mixture was made (pH = 2 to 3) by the addition of concd HCl thereto and extracted with EtOAc. After concentration, the residue was washed with hexane−EtOAc to give the object compound (15.5 g, 56%) as a white crystalline solid.

General Preparation for *N*-Hydroxysuccinimide Ester (3): *N*-(4-Fluorophenylsulfonyl)-L-valine *N*-Hydroxysuccinimide Ester. To a solution of the above compound (15.0 g, 55 mmol) and *N*-hydroxysuccinimide (7.6 g, 66 mmol) in THF (200 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (12.6 g, 66 mmol) in CH_2Cl_2 (200 mL) under an ice-cooled condition. The mixture was stirred for 4 h at room temperature. Then evaporation of solvent, EtOAc was added to the residue and the solution was washed with dilute HCl and sat. aq $NaHCO_3$. After concentration, the residue was washed with hexane−EtOAc to give the target compound (17.6 g, 87%) as a white crystalline solid.

General Procedure for Dipeptidyl Alcohol (4) Preparation: *N*-(4-Fluorophenylsulfonyl)-L-valyl-L-leucinol. To a solution of the above ester (2.0 g, 5.4 mmol) and L-leucinol (0.82 g, 7.0 mmol) in CH_2Cl_2 (50 mL) was added Et_3N (1.6 g, 16 mmol) and stirred for 2 h at room temperature. Then the reaction mixture was washed with dilute HCl and sat. aq $NaHCO_3$. After concentration, the residue was washed with hexane−EtOAc to give the target compound (1.9 g, 94%) as a white crystalline solid.

General Procedure for Dipeptidyl Aldehyde Preparation: *N*-(4-Fluorophenylsulfonyl)-L-valyl-L-leucinal (8). To a solution of the above alcohol (10 g, 27 mmol) in DMSO (100 mL) and CH_2Cl_2 (50 mL) was added Et_3N (16 g, 160 mmol). Purified sulfur trioxide−pyridine complex (17 g, 110 mmol) in DMSO (70 mL) was added thereto, and then the mixture was stirred for 40 min at 0−5 °C. EtOAc was added to the mixture and washed with dilute HCl and sat. aq $NaHCO_3$. After concentration, the residue was recrystallized from EtOAc to give **8** (1.9 g, 17%).

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Supporting Information Available: Complete experimental details, all biological test protocol and data, and elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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