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European Journal of Medicinal Chemistry 44 (2009) 1331–1334
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Short communication

Design and synthesis of calpain inhibitory 6-pyridone 2-carboxamide derivatives

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Received 4 August 2007; received in revised form 9 February 2008; accepted 12 February 2008
Available online 7 March 2008

Abstract

Excessive calpain activation contributes to serious cellular damage in many pathological conditions. The involvement of μ-calpain in neurological disorders such as, stroke and Alzheimer's disease has attracted considerable interest in the use of calpain inhibitors as therapeutic agents. 6-Pyridone 2-carboxamides derived from ketoamides were synthesized as conformationally constrained structures resembling the well known peptidic μ-calpain inhibitor, MDL 28,170, and their μ-calpain inhibitory activities were evaluated. Of the compounds synthesized, compound **2a**, which has a primary amide at warhead region of the inhibitor most potently inhibited μ-calpain with an IC_{50} value of 2.81 ± 1.26 μM, which is ca. 40-fold less than that of MDL 28,170.

Keywords: Calpain inhibitor; Stroke; Conformational restriction; 6-Pyridone

1. Introduction

Calpains are calcium-dependent, intracellular proteolytic enzymes and are found in many cells. Calpains are referred to as cysteine proteases because a cysteine residue is involved in the catalytic process [1]. The calpains are involved in a variety of calcium-regulated cellular processes, such as, signal transduction, cell proliferation and differentiation, apoptosis. However, excessive calpain activation causes serious cellular damage or even cell death. Moreover, the suggested involvement of μ-calpain in neurological disorders such as cardiovascular dysfunction [2], stroke [3], and Alzheimer's disease [4] has attracted much research interest in the use of calpain inhibitors as potential therapeutics. Most of the known calpain inhibitors bind to the catalytic center in a competitive manner and are derived from small peptides, in particular dipeptide aldehydes such as Z-Val-Phe-H (**1**, MDL 28,170), which are structurally related to the cleavage site of calpain substrates [5,6]. However, MDL 28,170 suffers from the disadvantages such as nonselectivity and excessive metabolism due to its peptide character and high reactivity of its aldehyde moiety [7]. Therefore, recent studies have mainly focused on the design of less peptide-like calpain inhibitors resulting in the synthesis of proline sulfonamides [8], benzoyl ketoamides [9] and peptidyl hydrazones [10]. In addition, we recently described the use of chromone and quinolinone rings as new scaffolds for μ-calpain inhibitors [11,12].

The use of conformationally restricted molecules to better understand or improve the activity of a parent molecule is a common theme in medicinal chemistry. The syntheses of conformationally restricted analogues of a lead compound often result in improved specific binding affinity for the target molecule [13–15]. For example, conformationally restricted structures

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doi:10.1016/j.ejmech.2008.02.023

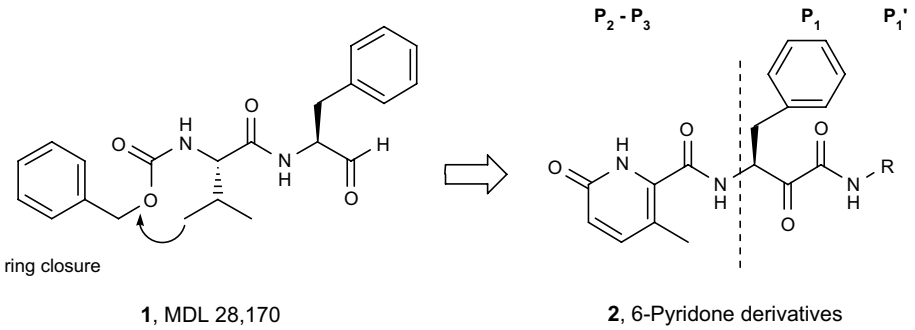
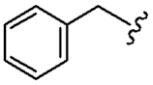
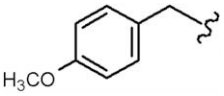
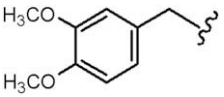
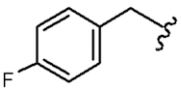
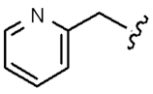
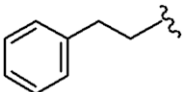
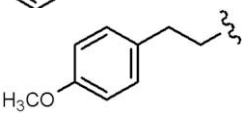
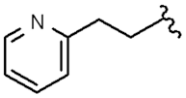


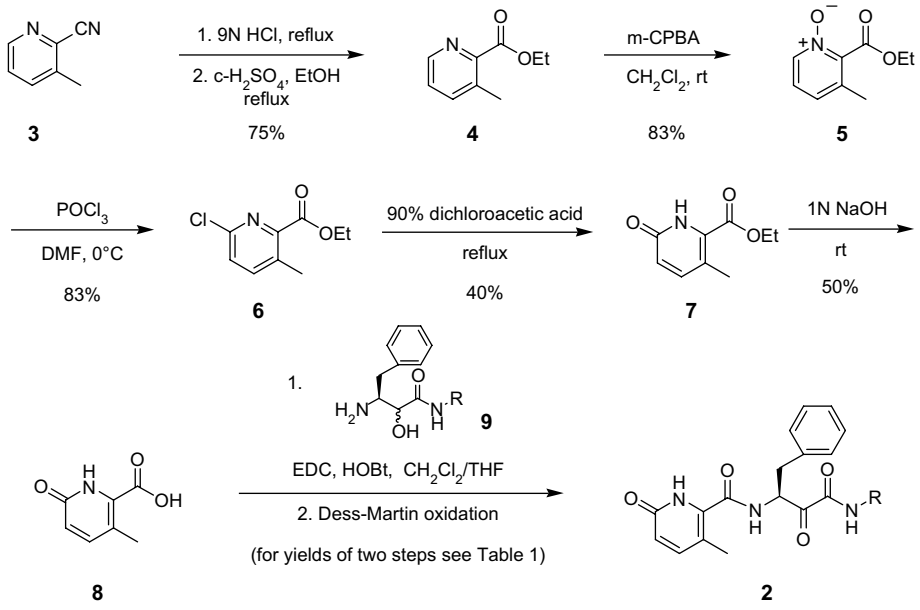
Fig. 1. Design of new μ-calpain inhibitors.

of peptidic μ-calpain inhibitors such as 2,3-methanoleucine [16], and piperidine [17], and leucinal and valyl side chains-linked to an eight-membered ring [18] have been described, some of which have interesting pharmacological properties.

We have also focused on restricting of conformational flexibility to understand the interaction between inhibitors and the active site of μ-calpain to facilitate the design of more potent inhibitors. Because it is well known that the P_1 residue of

Table 1
Coupling and oxidation yields and the μ-calpain inhibitory activities of **2a–l**

Inhibitors	R–	Yields (%)		μ-Calpain inhibition IC_{50} (μM)
		EDC coupling	Oxidation	
2a	H–	50	35	2.81 ± 1.26
2b	H_3C–	36	63	91.42 ± 7.00
2c		39	47	40.17 ± 3.77
2d		79	58	>100
2e		43	62	64.61 ± 17.45
2f		39	15	6.19 ± 0.43
2g		83	43	35.26 ± 10.33
2h		76	73	22.13 ± 0.21
2i		32	24	84.75 ± 0.21
2j		42	58	7.41 ± 0.49
2k		35	57	6.19 ± 0.41
2l		56	44	34.01 ± 5.47
1				0.07 ± 0.01



Scheme 1.

inhibitors should possess large hydrophobic groups like phenylalanine to achieve optimal *in vitro* activity [19], we decided to modify the P_2–P_3 position of inhibitors by restricting the conformations of the benzyloxycarbonyl and valyl groups in **1** by joining the valyl and carbamate oxygen groups in the peptidic inhibitor **1** to a 6-pyridone ring (Fig. 1). This structural modification restricts the conformational flexibility of the amide linkage and possibly makes the resulting cyclic amide linkage more resistant to proteolytic cleavage due to the non-natural amide linkage of a 6-pyridone ring [16,18]. In this report we describe the synthesis and μ-calpain inhibitory activities of 6-pyridone 2-carboxamides **2** incorporating a 6-pyridone ring at the P_2–P_3 position of inhibitors. Instead of an aldehyde moiety in **1**, a ketoamide was used as a warhead since several ketoamide-derived inhibitors showed comparable activity and improved metabolic stability [20]. To investigate the influence of substituents on inhibitory activities, we modified the amide region (R_1) of the inhibitors to prove the interaction at the P_1'-subsite of inhibitors with the enzyme.

2. Results and discussion

The cyano group in 3-methyl-picolinonitrile (**3**) was hydrolyzed by refluxing in 6 N HCl and then converted to ethyl ester by refluxing in ethanol in the presence of *c*-H_2SO_4 to provide **4**. Ethyl 2-picolinate **4** was reacted with *m*-chloroperoxybenzoic acid to form the corresponding *N*-oxide **5**, which when treated with phosphoryl chloride gave ethyl 6-chloropicolinate **6**. Hydrolysis of **6** with 90% dichloroacetic acid in H_2O gave 6-pyridone 2-carboxylic acid ethyl ester (**7**), the ester group of which was hydrolyzed using 1 N NaOH. Neutralization of the reaction mixture with cation exchange resin (Amberlite IR-120) followed by filtration of the resulting white solid and drying gave 6-pyridone 2-carboxylic acid **8** [21].

Compound **8** was coupled to various 3-amino-2-hydroxybutanoic acid amides (**9a**–**l**) [11], the P_1 building blocks, using EDC/HOBt to afford hydroxyamides **10a**–**l**, which were subsequently transformed into the 6-pyridone 2-carboxylic acid derivatives **2a**–**l** by oxidation under Dess–Martin periodinane conditions. The yields of coupling and oxidation reactions are summarized in Table 1 [22] (Scheme 1).

The prepared 6-pyridone 2-carboxamide derivatives **2a**–**l** were evaluated for inhibition of μ-calpain using human calpain I, which isolated from erythrocytes and Suc-Leu-Tyr-AMC as the fluorogenic substrate [23]. The results are summarized in Table 1. MDL 28,170 (**1**) was also tested for comparison. The primary amide **2a** showed the most potent inhibition of μ-calpain ($IC_{50} = 2.81$ μM), and the potencies reduced when the alkylamide group was substituted. When *N*-alkylaryl substituents were incorporated in the P_1' region, moderate inhibitory activities were observed. Notably, compounds containing a 4-methoxy substituent at the benzene ring (**2f**, **2k**) were more potent than other (un)substituted *N*-alkylaryl derivatives. On the other hand, the incorporation of a heterocycle (**2i**, **2l**) or substitution of the fluoro group (**2h**) decreased inhibitory activity. However, compounds **2a**, the most potent μ-calpain inhibitor of the series, were 40-fold less potent than MDL 28,170 (**1**), which suggests that the strategy involving the rigidifying of the valyl group in **1** needs to be revised to promote binding at the catalytic site.

3. Conclusions

6-Pyridone 2-carboxamide derivatives were synthesized as conformationally constrained structural variants of MDL 28,170 in an effort to understand the structural requirements for inhibitor binding to the active site of the calpain. Of the derivatives synthesized, 6-pyridone 2-carboxamide **2a**, which

contains a primary amide, most potently inhibited μ-calpain with an IC_{50} value of 2.81 ± 1.26 μM. It was less potent than that of the parent compound, MDL 28,170 (**1**). These results of the activity testing of **2**, which has limited conformational flexibility in the P_2–P_3 region of the inhibitors encourage us to further investigate the molecular recognition requirements of the active site of μ-calpain.

Acknowledgements

This research was supported by the Mid-Term Technological Development Project funded by the Korean Ministry of Commerce, Industry and Energy (grant no. 10027898-2007-22).

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[22] Spectral data of selected compounds. Compound **6**: ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.57 (1H, d, $J = 8.4$ Hz, *H*-4), 7.35 (1H, d, $J = 8.4$ Hz, *H*-5), 4.45 (2H, q, $J = 7.2$ Hz, $-OCH_2CH_3$), 2.54 (3H, s, $-CH_3$), 1.45 (3H, t, $J = 7.2$ Hz, $-OCH_2CH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.2, 148.4 148.0, 142.5, 133.6, 126.6, 62.0, 19.1, 14.3. Compound **7**: ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.29 (1H, d, $J = 9.2$ Hz, *H*-4), 6.70 (1H, d, $J = 9.4$ Hz, *H*-5), 4.39 (2H, q, $J = 7.4$ Hz, $-OCH_2CH_3$), 2.40 (3H, s, $-CH_3$), 1.40 (2H, t, $J = 7.4$ Hz, $-OCH_2CH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.7, 161.5, 145.6, 129.9, 126.3, 121.4, 62.7, 18.3, 14.2. Compound **8**: ^{1}H NMR (200 MHz, D_2O) δ 7.48 (1H, d, $J = 9.2$ Hz, *H*-4), 6.48 (1H, d, $J = 9.2$ Hz, *H*-5), 2.17 (3H, s, $-CH_3$); ^{13}C NMR (75 MHz, D_2O) δ 167.9, 162.7, 147.7, 137.6, 119.5, 118.3, 16.0. Compound **2a**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67 (1H, br s, $-NH$), 8.12 (1H, s, $-NH$), 8.87 (1H, s, $-NH$), 7.47 (1H, d, $J = 8.4$ Hz, $-COCHCH-$), 7.29–7.18 (7H, m, aromatic), 6.36 (1H, d, $J = 8.4$ Hz, $-COCHCH-$), 5.46 (1H, m, $-NHCHCH_2Ph$), 3.22 (1H, dd, $J = 4.4$, 14.0 Hz, $-NHCHCH_2Ph$), 2.93 (1H, dd, $J = 8.8$, 14.0 Hz, $-NHCHCH_2Ph$), 2.19 (3H, s, $-C{=}C-CH_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 197.2, 165.7, 163.3, 161.4, 144.6, 137.9, 129.8, 129.2, 127.4, 55.8, 36.1, 18.1.
[23] Calpain inhibitory activity was assayed using a slight modification of a reported procedure [24]. Briefly, calpain-I (Calbiochem) from human erythrocytes and the fluorogenic calpain substrate (Suc-Leu-Tyr-7-amino-4-methylcoumarin, Calbiochem) were used as an enzyme and substrate, respectively. In a typical experiment, 1 μl of a compound solution at various concentrations, 25 μl of 1 mM calpain substrate, 10 μl of 1 M NaCl and 11 μl of 1 mM $CaCl_2$ were added to each well of 96-well plates. All chemicals except for the compounds concerned were dissolved in 50 mM Tris–HCl (pH 7.5) buffer containing 1 mM dithiothreitol (DTT) and used immediately. The compounds were dissolved in DMSO, but the DMSO concentrations in the final experimental solutions did not exceed 1%. Incubations were initiated by adding 53 μl of 34 nM calpain and the mixture was incubated for 30 min at room temperature. After incubation, the fluorescence of the cleavage product, 7-amino-4-methylcoumarin, was measured using a spectrofluorimeter at $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm against a blank sample not containing calpain. The IC_{50} values were calculated using percent inhibitions of enzyme activity.
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