Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 2315–2319

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Calpain Inhibitors Based on the Quiescent Affinity Label Concept: High Rates of Calpain Inactivation with Leaving Groups Derived from *N*-Hydroxy Peptide Coupling Reagents

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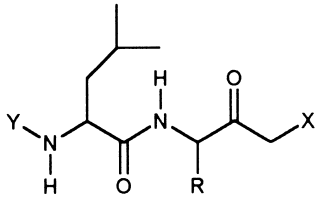
Received 26 May 2000; accepted 4 August 2000

Abstract—A series of irreversible inhibitors of recombinant calpain has been synthesized and their rates of inactivation have been evaluated against calpain and cathepsin B, respectively. The design of the inhibitors was based on the quiescent affinity label concept. By choosing the appropriate affinity group and by employing leaving groups derived from *N*-hydroxy coupling reagents, good inhibitors of calpain with high rates of inactivation have been identified. However, poor aqueous stability limits their therapeutic utility.

Introduction

The calpains are a family of cytosolic calcium-activated cysteine proteases, which exist in mammalian cells as both ubiquitous and tissue-specific forms. The best characterized members of the family are the ubiquitous enzymes calpain I and calpain II, heterodimeric proteases that contain different catalytic subunits but a common regulatory subunit, and which can be distinguished biochemically by the concentration of calcium required for activation.[1] The calpains have been postulated to be involved in pathology of several diseases, suggesting that calpain inhibitors might have clinical utility in a variety of disorders.[2] The design of inhibitors of calpain is being pursued from many directions, which are categorized into two distinct classes. Chemical entities comprising peptidyl aldehydes and α-ketoamides are reversible[3] inhibitors, while fluoromethylketones, diazomethylketones, acyloxymethyl ketones, sulphoniummethyl ketones, peptidyl epoxides (E-64), vinyl sulfones and haloacylhydrazines are irreversible[4] inhibitors of cysteine proteases. The design of effective irreversible inhibitors of calpain is challenging, as the enzyme is inert towards many of the different classes of known cysteine protease inhibitors. Generally a relatively long peptide sequence (typically a tripeptide) or a dipeptide with a modified capping group[4g] is required to attain satisfactory rates of calpain inactivation Herein, we report on calpain inhibitors utilizing the *quiescent affinity label concept,*[5] which show high rates of inactivation of recombinant calpain with unconventional leaving groups derived from *N*-hydroxy peptide coupling reagents.

We have used a dipeptidyl methyl ketone (**1**) that bears a leaving group (**X**) at the α-position as our basic molecular skeleton.[6] The peptidyl segment serves as the affinity group, while the leaving group can be systematically varied to explore and optimize the rate of enzyme inactivation. Calpain becomes inactivated as its catalytic thiol group forms a covalent bond to the inhibitor through displacement of the leaving group via direct or indirect attack at the carbon bearing the leaving group.[5] Although Krantz and co-workers have shown that peptidyl acyloxymethyl ketones work extremely well as inhibitors of cathepsin B, they provide only a modest degree of time-dependent inactivation of chicken smooth muscle calpain II, even with an affinity group optimized for calpain.[5f]



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In their paper,[5f] Krantz et al. discount the possibility that the poor reactivity of calpain towards acyloxymethyl

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0960-894X/00/$ - see front matter
PII: S0960-894X(00)00451-0

ketones was due to the weak nucleophilicity of the calpain active site thiolate group, since it is quite reactive towards peptidyl fluoromethyl ketones. Intolerance of the steric bulk of substituted benzoates or the absence of a residue appropriately positioned in the calpain active site to protonate the leaving group was postulated to result in the lack of reactivity of calpain toward benzoate-based inhibitors. We envisioned that steric incompatibility of benzoate leaving groups in the calpain active site could be the principal problem. We chose to explore this by choosing departing groups with inherent conformational and binding[7] properties different from the benzoates. Such properties might be helpful in pointing the leaving group toward a more accommodating region of the enzyme or could place it in the recognition domain of a proton-donating group in the active site, which would facilitate its expulsion as a neutral species. After considerable experimentation, we identified a novel class of leaving groups derived from *N*-hydroxy peptide coupling reagents (Table 1). The anions of *N*-hydroxy succinimides (HOSu) and benzotriazoles (HOBt) are not conventional leaving groups in organic synthesis (i.e., to facilitate nucleophilic attack at an isolated sp^3 carbon). However, their role as leaving groups in active esters during peptide synthesis are well known. Moreover, like substituted benzoates they have low pK_a values[8] (pK_a value of HOBt is 4.3), which has been postulated to correlate the rate of inactivation of cathepsin B by peptidyl acyloxymethyl ketones.[5]

Chemistry

The synthesis of inhibitors was accomplished from peptidyl bromomethyl ketones. A representative example is shown in Scheme 1. The mixed anhydride of dipeptide acid, Z-Leu-Phe-OH (**2**) was treated with diazomethane to generate the diazoketone, which was decomposed in the presence of HBr/HOAc to generate bromoketone **3**. Reaction of **3** and HOBt in DMF with silver(I) oxide or KF as a base provides inhibitor **1d**, which was purified by crystallization.[9]

Biology

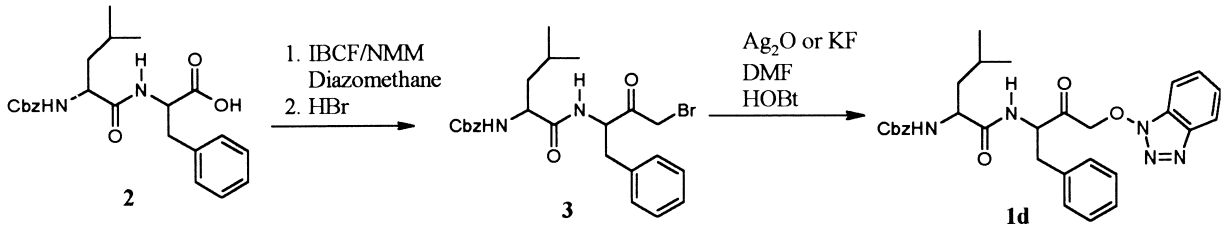
The inhibitory activity of the compounds **1a–j** was determined using recombinant human calpain I, prepared as described by Meyer and co-workers.[10] Second-order rate constants for inactivation were determined by analysis of the progress curves obtained in the presence of substrate (Suc-Leu-Tyr-MNA)[11] and inhibitor. Assays for human liver cathepsin B (Calbiochem) were performed by a similar procedure employing published assay conditions.[12]

Results and Discussion

Table 1 records the second-order rate constants for inactivation of calpain and cathepsin B. The inhibitors **1a–c** containing a succinimide or a substituted succinimide analogue as a leaving group were weak inactivators, with **1a** having the highest rate of inactivation. However, the benzotriazoloxy **1d** was an extremely rapid time-dependent inactivator of calpain. Inhibitor **1d** has one of the highest rates of inactivation utilizing a dipeptide affinity group reported so far for inhibitors of calpain. For comparison, **1e**, a fluoromethyl ketone bearing the same affinity group shows a rate of calpain inactivation of 136,300 M^{-1} s^{-1}.[4g] By modifying the capping group of **1e** or by using a tripeptide affinity group, inhibitors with high rates of calpain inactivation (up to 290,000 M^{-1} s^{-1}) have been made.[4g] Other examples with high rates of calpain inactivation are from a dipeptidyl methyl phosphate[13] (365,000 M^{-1} s^{-1}), a tripeptidyl diazomethyl ketone[4a] (230,000 M^{-1} s^{-1}) and a tripeptidyl sulfonium methyl ketone[5f] (>200,000 M^{-1} s^{-1}). The inactivation by **1d** appears to be irreversible, since no catalytic activity was recovered following removal of inhibitor by repeated ultrafiltration through a Centicon 30. Based on initial velocities, a K_i of approximately 35 nM was determined for **1d**, indicating that it binds well to calpain.

Modification of the P_1 position of the inhibitor's peptidyl affinity group resulted in compounds **1f** and **1g**, which display a decrease in inactivation rate analogous to that observed with the fluoromethyl ketones[4g] corresponding to **1d** and **1g**. Increasing the length of the affinity group to a tripeptide (**1h**) yielded an approximately twofold increase in the rate of inactivation. Compounds **1i** and **1j** were prepared by incorporating the related peptide coupling reagents 7- and 4-HOAt,[14] respectively. Both inhibitors maintain a respectable level of activity toward calpain.

It seems that the role of the N–O bond, presumably its conformation,[15] is extremely crucial for optimal calpain inactivation rate. Replacement of the benzotriazoloxy group with the corresponding benzotriazolyl (**1k**) provides evidence in that respect. Though benzotriazolyl anion is considered as an extremely good leaving group in organic synthesis,[16] compound **1k** was found to be a poor inhibitor of calpain. From comparison of the

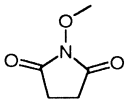
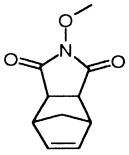
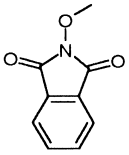
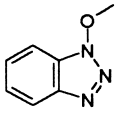
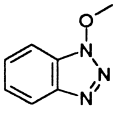
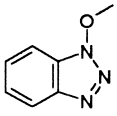
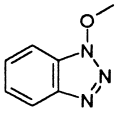
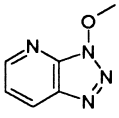
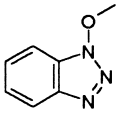
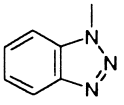


Scheme 1.

results from **1d** with **1k**, we speculate that, apart from a good degree of departing and binding[7] potential for the leaving groups and contributions from the peptidyl affinity group, the origin of high reaction rates for compounds such as **1d** could be ascribed to the role of the N–O bond which is believed to play a pivotal role in providing a stereoelectronically favorable orientation of the leaving group in the calpain active site.

The selectivity of the benzotriazoloxy class of inhibitors with respect to inhibition of cathepsin B is also shown in Table 1. Although these compounds are good cathepsin B inhibitors, the degree of selectivity can be influenced by the nature of the peptide affinity group, as evidenced by **1d** versus **1f**. The nature of the benzotriazoloxy group appears to have a less significant effect on selectivity, at least for this limited set of analogues.

Table 1. Inhibition of calpain and cathepsin B by inhibitors **1a–k**

Inhibitor	X	Y	R	Calpain I $k_{obs}/[I](M^{-1}\ s^{-1})$	Cathepsin B $k_{obs}/[I](M^{-1}\ s^{-1})$	Calpain/cathepsin B
1a		Cbz	Benzyl	5800	[a]	—
1b		Cbz	Benzyl	1000	6900	0.14
1c		CBz	Benzyl	[b]	[a]	—
1d		Cbz	Benzyl	320,000	118,000	2.7
1e	F	Cbz	Benzyl	136,300[c]	300[c]	454.3
1f		CBz	*i*-Butyl	175,000	19,400	9.0
1g		Cbz	Ethyl	63,000	443,000	0.14
1h		Cbz-Leu	Benzyl	524,000	[b]	—
1i		Cbz	Benzyl	184,000	107,000	1.7
1j		Cbz	Benzyl	57,000	85,500	0.67
1k		Cbz	Benzyl	[d]	[a]	—

[a]Not determined.
[b]32% inhibition at 10 μm following 5 min incubation.
[c]See ref 4g.
[d]83% inhibition at 10 μm following 5 min incubation.

A limitation of the peptidyl benzotriazoloxy methyl ketones is their modest stability in aqueous solution at neutral pH.[17] Estimates of stability were obtained by preincubating inhibitor in assay buffer for various times. Progress curve experiments were then initiated by the addition of calpain, substrate and calcium, and the resulting inactivation rates were used as a measure of remaining inhibitor concentration. The half-life for compound **1d** was approximately 5 min. As a control, the fluoromethyl ketone **1e** was tested by the above method and shown to demonstrate no measurable decomposition over 60 min. There was no correlation between the inactivation rates for calpain and cathepsin B and the half-life for loss of inhibitor. Because of the limited stability of the series, all inactivation rates were determined as rapidly as possible after addition of inhibitor to the assay solution. Due to poor aqueous stability, the measured inactivation rates for **1d** and other analogues (**1f–j**) represent lower limits.

In conclusion, we have synthesized a novel class of irreversible inhibitors of calpain containing a relatively short dipeptide affinity groups. High inactivation rates were obtained against two cysteine proteases through manipulation of the peptide address and recognition elements within the leaving group. However, poor aqueous stability of those compounds severely limits their therapeutic utility.

Acknowledgements

The authors would like to thank Dr. Jeffry Vaught for his support and encouragement. We would also like to express our sincere thanks to Mrs. Shobha E. Senadhi and Dr. Satish Mallya for carrying out enzyme assays and to Drs. Chakrapani Subramanyam, Ming Tao and Ron Bihovsky for their contributions towards stability studies.

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6. Leucine at p2 of the peptide affinity group was selected as calpain prefers a Val or Leu at p2. See ref 1.

7. The leaving group of the inhibitor might be providing additional prime site binding interactions with the enzyme. Inhibitors of cathepsin K (a cysteine protease) with prime site binding have been identified. See Yamashita, D. S.; Smith, W. W., Zhao, B.; Janson, C. A.; Tomaszek, T. A.; Bossard, M. J.; Levy, M. A.; Oh, H.-J.; Carr, T. J.; Thompson, S. K.; Ijames, C. F.; Carr, S. A.; McQueney, M.; D'Alessio, K. J.; Amegadzie, B. Y.; Hanning, C. R.; Abdel-Meguid, S.; DesJarlais, R. L.; Gleason, J. G.; Veber, D. F. *J. Am. Chem. Soc.* **1997**, *119*, 11351.

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11. MNA = β-methoxy naphthylamine.

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monitored until there was no further product generated in inhibitor-containing assays. Inhibitor concentrations were at least 10-fold greater than the enzyme concentration in all cases. Values of k_{obs}, the pseudo first-order rate constant for inactivation, were calculated from plots of fluorescence versus time by nonlinear regression (Sigma Plot) to the exponential eq (1)[5b]

$$y = Ae^{(-k_{obs}*t)} + B \quad (1)$$

where y is the fluorescence at time t (F_t), A is the amplitude of the reaction (F_0-F) and B is the maximal amount of the product formed when the enzyme is completely inactivated (F). The apparent second-order rate constant for inactivation was calculated from the slope of a plot of k_{obs} versus inhibitor concentration as $(k_{obs}/I)*(1 + S/K_m)$, correcting for the effect of substrate on the inactivation rate. (b) Rates of inactivation of cathepsins B were determined under the assay conditions described by Krantz et al.[5b]

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15. For N–O bond conformational barrier and its role in biological systems: Kahne et al. have shown via computational methods that the unusual N–O bond in calicheamicin with its distinctive torsional angle preference holds the two halves of the molecule in a shape suitable for DNA binding. Kahne, D.; Gupta, V.; Gange, D.; Walker, S. *J. Am. Chem. Soc.* **1994**, *116*, 3197.
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17. We postulate an elimination pathway for the decomposition of these compounds (**1d** and others) in aqueous media which is evidenced by the detection of benzotriazole (**5**) as one of the products. Though the major elimination product, the keto-aldehyde **4**, could not be isolated, it has been independently prepared and was found not to be a time-dependent inactivator of calpain. Therefore, it is reasonable to assume that *intact* **1d** as well as **1f–j** are inactivators of calpain and cathepsin B.