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Bioorganic & Medicinal Chemistry Letters
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Bioorganic & Medicinal Chemistry Letters 14 (2004) 685–687

Peptidyl aldehydes as slow-binding inhibitors of dual-specificity phosphatases

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Received 15 October 2003; revised 13 November 2003; accepted 14 November 2003

Abstract—Peptidyl aldehydes were tested for inhibition of dual-specificity phosphatases VH1 and VHR. The most potent compound, cinnamaldehyde–Gly–Glu–Glu (Cinn–GEE), acted as a slow-binding inhibitor with K_I* values of 18 and 288 μM against VH1 and VHR, respectively.

Protein phosphorylation is one of the most important posttranslational modifications and regulates many cellular processes such as cell growth and differentiation. The level of phosphorylation is controlled by the opposing action of protein kinases and protein phosphatases. There are three types of protein phosphatases. Protein tyrosine phosphatases (PTPs) catalyze the hydrolysis of phosphotyrosine (pY) residues in proteins, whereas protein serine/threonine phosphatases remove the phosphoryl group from phosphoseryl and phosphothreonyl residues. In addition, a third class of dual-specificity phosphatases (DSPs) are capable of hydrolyzing both phosphoseryl/phosphothreonyl and phosphotyrosyl (pY) residues. Like PTPs, DSPs have been shown to be involved in important cellular events, such as intracellular signaling. For examples, VH1 (Vaccinia open reading frame H1) phosphatase,[1] which was the first DSP identified, can reverse Stat-1 activation in vaccinia virus-infected cells.[2] Another DSP, human VHR (VH1 related) phosphatase,[3] was shown to play a critical role in intracellular signaling mediated by mitogen-activated protein (MAP) kinase.[4] Despite significant recent progresses, the precise cellular roles of many DSPs are still largely unknown. Therefore, specific inhibitors against DSPs may provide useful probes for studying their physiological functions and potential therapeutic agents. Unfortunately, there have been relatively few reports[5] on DSP inhibitors, most of which were identified from high-throughput screening.

Our laboratory is engaged in an ongoing effort on the development of neutral phosphotyrosine mimetics as inhibitors of PTPs and Src homology 2 (SH2) domains.[6–8] We recently reported that certain peptidyl aldehydes, such as the adduct of cinnamaldehyde and tripeptide Gly–Glu–Glu–NH_2 (Cinn–GEE) (compound **2** in Fig. 1), act as slow-binding inhibitors of PTPs[7] and SH2 domains.[8] Mechanistic studies revealed that inhibitor **2** covalently modifies a conserved arginine in the pY-binding pockets (Arg-221 in PTP1B and Arg-βB5 in SH2 domains) and forms a reversible imine/enamine adduct with the PTP or SH2 domain (Fig. 2). Because DSPs share the same signature motif (HCX_5RS/T),[9] active-site structure,[4b] and catalytic mechanism as PTPs,[10] we reasoned that the peptidyl aldehydes might also inhibit DSPs by modifying the arginine in the

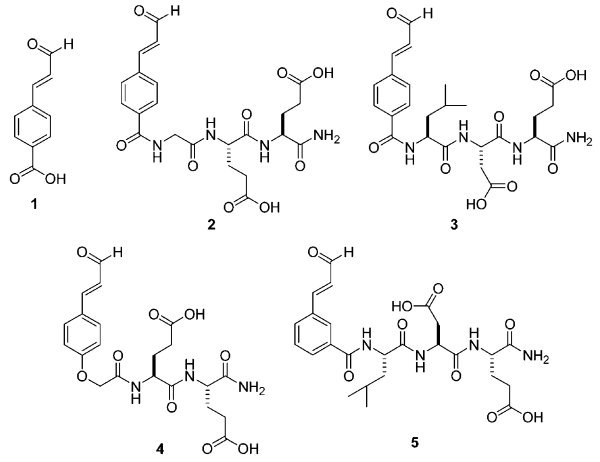

Figure 1. Structures of peptidyl cinnamaldehydes used in this work.

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doi:10.1016/j.bmcl.2003.11.027

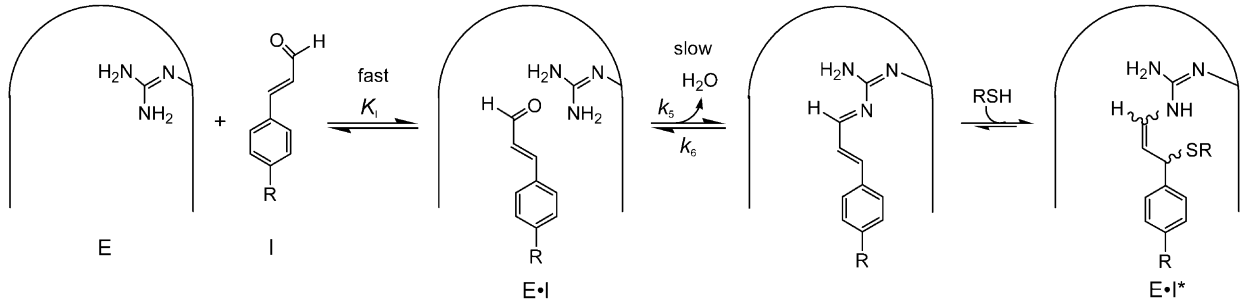

Figure 2. Proposed mechanism of inhibition of PTPs and SH2 domains by cinnamaldehyde derivatives. RSH, β-mercaptoethanol.

signature motif. Here, we show that the aldehydes are indeed DSP inhibitors, suggesting that cinnamaldehyde is a general, neutral pY mimetic.

We first tested *p*-carboxycinnamaldehyde (**1**) against DSPs VH1 and VHR.[11] While compound **1** showed only ~40% inhibition of VHR at 2.5 mM, it was more potent against VH1 with an IC_{50} value of ~1 mM, which is comparable to that against PTP1B (IC_{50} = 970 μM).[7] Next, the peptidyl aldehyde **2** was tested against the enzymes. Aldehyde **2** behaved as a slow-binding inhibitor toward VH1, as evidenced by the biphasic reaction progress curves (Fig. 3a) and the time-dependent reactivation of inhibited enzyme upon dilution (Fig. 3b). The inhibition kinetics can be described by the equation:

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E \cdot I \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E \cdot I^*$$

where K_I is the equilibrium constant for the formation of the initial E · I complex, whereas k_5 and k_6 are the forward and reverse rate constants for the interconversion between the E · I complex and the final E · I* complex. The overall equilibrium constant (K_I*) is described by $K_I^* = K_I k_6/(k_5 + k_6)$ and represents the overall potency of the inhibitor. The K_I and K_I* values of aldehyde **2** against VH1 were 190 ± 17 and 18 ± 3 μM, respectively.[12] Fitting of the reactivation curve in Figure 3b against the equation[13]

$$Abs_{405} = v_s[t - (1 - e^{-k_6 t})/k_6]$$

produced the reactivation rate constant (k_6 = 0.68 min^{-1}). The forward rate constant for conversion of E · I to E · I* was calculated using the equation $K_I^* = K_I k_6/(k_5 + k_6)$ (k_5 = 6.6 min^{-1}). Aldehyde **2** also inhibited VHR, but with much lower potency. The K_I* value for VHR was 290 ± 110 μM,[7] whereas the K_I, k_5, or k_6 values could not be accurately determined, due to the lower potency.

To determine whether aldehyde **2** is active site-directed, hydrolysis reactions were carried out at a fixed inhibitor concentration and varying substrate concentrations [1.2–30 mM *p*-nitrophenyl phosphate (*p*NPP)]. An inverse correlation was observed between the k_{obs}, the pseudo-first-order rate constant for onset of inhibition, and *p*NPP concentration (Fig. 4). This result indicates that aldehyde **2** binds to the active site of VH1. The mechanism of inhibition was further investigated using

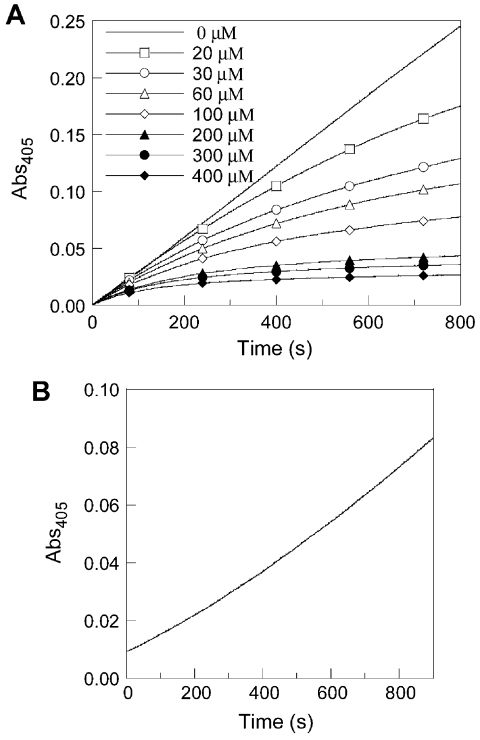

Figure 3. Slow-binding inhibition of VH1 by aldehyde **2**. (A) Hydrolysis of *p*NPP (5 mM) by VH1 (0.1 μM) in the presence of indicated amounts of aldehyde **2**. The reactions were initiated by the addition of VH1 as the last component. (B) Hydrolysis of *p*NPP by reactivated VH1. VH1 (0.2 μM) was preincubated with aldehyde **2** (260 μM) for 3 h before being diluted 10-fold into a reaction solution containing 5 mM *p*NPP.

^{13}C-labeled **2** and ^1H–^{13}C heteronuclear single-quantum correlation spectroscopy as previously described for PTP1B[7] and SH2 domains.[8] Unfortunately, these experiments failed to provide any mechanistic insight, due to difficulty in preparing highly concentrated VH1/aldehyde **2** samples for the NMR experiments. Attempts to capture the covalent adduct through reduction with sodium cyanoborohydride followed by detection by mass spectrometry also failed, as was the case with PTP1B.[7] However, on the basis of the similarities in their active-site structures and the inhibition kinetics, we propose that aldehyde **2** inhibits DSPs via a mechanism similar to that of PTPs (Fig. 2), by covalently modifying the conserved active-site arginine (Arg-116 in VH1). The failure to be reduced by sodium cyanoborohydride is consistent with the formation of an enamine adduct, as previously observed for PTPs.[7]

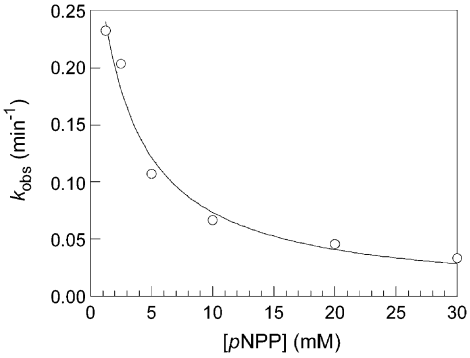

Figure 4. Competition between aldehyde **2** and *p*NPP for binding to VH1. Reactions were initiated be the addition of VH1 (0.2 μM) to solutions containing a fixed amount of **2** (100 μM) and varying *p*NPP concentrations (1.2–30 mM). The k_{obs} values were obtained by fitting individual time courses to the equation: $[p] = v_s t + [(v_i - v_s)/k_{obs}](1 - e^{-k_{obs}t})$.[13] The solid line is a best fit to the data according to the equation: $k_{obs} = k_{max}/(1 + [p\text{NPP}]/K_M)$.

Comparison of the potency of compounds **1** and **2** against VH1 revealed that the addition of tripeptide Gly–Glu–Glu improved the affinity by >50-fold. To determine whether other tripeptides could produce the same effect, we tested several other peptidyl cinnamaldehydes (Fig. 1 compounds **3**–**5**), which had previously been selected from a combinatorial library against PTP1B (unpublished data), against VH1. Despite their structural similarities to aldehyde **2**, these compounds showed much lower potency against VH1 than **2** (Fig. 5). These data indicate that specific peptide structures (or peptidomimetic structures) and a proper linkage between the peptide and the cinnamaldehyde are both necessary to generate potent peptidyl cinnamaldehyde inhibitors of VH1.

In summary, we have demonstrated that aldehyde **2** is a slow-binding inhibitor of moderate potency against DSPs. Together with our earlier reports, our results suggest that cinnamaldehyde could be used as a general pY mimetic for inhibition of pY-binding proteins (e.g., SH2, PTPs, and DSPs). More potent and specific inhibitors against these pY-binding proteins may be developed by designing and screening combinatorial libraries of peptidyl cinnamaldehydes. Such work is already underway in this laboratory.

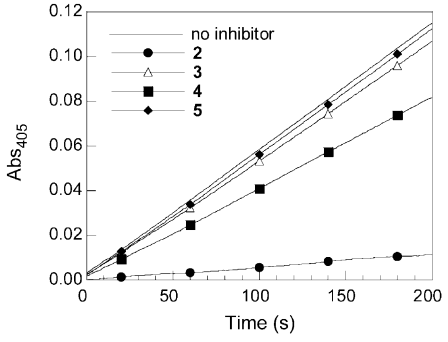

Figure 5. Inhibition of VH1 by peptidyl aldehydes **2**–**5**. VH1 (0.2 μM) was preincubated with each inhibitor (40 μM) in the reaction buffer[11] for 1 h at room temperature and the reaction was then initiated by the addition of 5 mM *p*NPP (final concentration) and monitored on a UV–Vis spectrophotometer.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (GM62820). We thank Drs. John Denu and Kun-liang Guan for providing the plasmids for overproduction of VHR and GST-VH1.

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11. Enzyme assays were performed as follows. A typical reaction (total volume 1.0 mL) contained 50 mM imidazole (pH = 7.0), 1 mM EDTA, 0.05% (v/v) β-mercaptoethanol, 5% DMSO, 0.1–0.2 μM VH1 (or 0.5–1 μM VHR), and 0–2700 μM inhibitor. After incubation of the enzyme with the inhibitor for 1 h at room temperature, the reaction was initiated by the addition of 5 mM *p*NPP. The reaction progress was monitored at 405 nm on a Perkin–Elmer UV–Vis spectrophotometer.
12. To determine the initial equilibrium constant, K_I, a typical assay reaction (1.0 mL) contained 50 mM imidazole (pH = 7.0), 1 mM EDTA, 0.05% (v/v) β-mercaptoethanol, 5% DMSO, 5 mM *p*NPP, and 0–400 μM inhibitor **2**. The reaction was initiated by the addition of VH1 (final 0.1 μM) as the last component and monitored continuously at 405 nm on a spectrophotometer. The initial rates obtained from the early regions of the reaction progress curves (0–60 s) were fitted to the Michaelis–Menten equation to give the K_I value. The K_I* value was determined as described above, except that pNPP was added as the last component, after the other components had been preincubated for 1 h. The k_6 value was determined from the reactivation experiment as described in the text. All measurements were performed at room temperature.
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