**Note**

**Inhibitory Effects of Alcohols on the Activity of Human Matrix Metalloproteinase 7 (Matrilysin)**

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Aliphatic alcohols inhibited the activity of human matrix metalloproteinase 7 (matrilysin) competitively with $K_i$ of 6.1–19.4% (v/v) or 0.66–4.80 M. From the relationship between the structures of alcohols and their $K_i$ values, alcohols are considered to bind the hydrophobic S1’ subsite most plausibly, and the size of the pocket was estimated to be large enough to accommodate the length of 1-butanol (4-carbon chain) and the bulk of tertiary alcohols. Alcohols might be suitable probes for exploring the active-site geometry of enzymes.

**Key words:** aliphatic alcohol; competitive inhibition; enzyme inhibitor; matrilysin; matrix metalloproteinase

Matrix metalloproteinases (MMPs) comprise a family of zinc endopeptidases that take part in both normal biological and pathological processes and are believed to play a major role in degrading extracellular matrix components. It is known that MMPs have a large substrate-binding site consisting of eight subsites (S4, S3, S2, S1, S1’, S2’, S3’, and S4’ according to the nomenclature of Schechter and Berger). Most MMPs share a similar preference for amino acid residues with aliphatic side-chains in the S1’ subsite, which is a well-defined hydrophobic pocket with different depth. The preference at the S1’ subsite is considered to determine the specificity of the peptide bond cleavable by MMPs. Matrilysin (EC 3.4.24.23) is one of the smallest members of the MMP family; its active form consists of a catalytic domain of only 19 kDa. Matrilysin, together with other MMPs, has been implicated in the invasion and metastasis of several tumor cells. The development of potent and selective inhibitors is desired. The S1’ subsite of matrilysin is shallower than those of many other MMPs, and most favorably accommodates a Leu residue in the substrates. We have reported that aliphatic alcohols inhibit a bacterial metalloproteinase, thermolysin, competitively with the inhibitor constant $K_i$ of 38–430 mM. It has been shown by X-ray crystallographic analysis that the side chain of alcohols is accommodated in the hydrophobic S1’ subsite of thermolysin. The S1’ subsite of thermolysin plays a leading role in determining the substrate specificity, and as well as that of matrilysin it prefers hydrophobic residues such as Leu, Ile, and Phe. In this study, we describe the inhibitory effects of nine aliphatic alcohols (Fig. 1) on matrilysin activity, and make a proposal as to the structure-activity relationship of alcohols for the inhibition of matrilysin.

The matrilysin-catalyzed hydrolysis of MOCa–PLGL(Dpa)AR, the Gly–t-Leu bond in which is cleaved by matrilysin, was performed in 50 mM HEPES buffer (pH 7.5) containing 10 mM CaCl$_2$ (standard buffer) with 0.6% DMSO at 25 °C. The hydrolysis was monitored by following the increase in the fluorescence intensity at 393 nm with excitation at 328 nm. All alcohols examined had the ability to inhibit matrilysin activity, and the inhibition was completely reversible at alcohol concentrations up to 15% methanol and ethanol, 10% 1-propanol, 2-propanol, and 2-methyl-2-propanol, and 5% 1-butanol, 2-butanol, 2-methyl-1-propanol, and 2-methyl-2-butanol (data not shown), which was confirmed by the alcohol dilution method described previously.

In the evaluation of the inhibitory manner of the alcohols, the hydrolysis of the substrate was monitored by HPLC rather than fluorometrically to avoid the absorptive quenching effect of the dinitrophenyl group at high concentrations of the substrate. In HPLC analysis, the reaction was performed in the standard buffer with 5% DMSO at 25 °C in order to dissolve a high enough concentration of the substrate to determine the catalytic constant ($k_{cat}$) and the Michaelis constant ($K_m$) separately. The plot of $[S]_o/v$ versus $[S]_o$ (Hanes–Woolf plot) in the absence and presence of 10% 1-propanol gave two parallel linear lines (Fig. 2A). The $k_{cat}$ and $K_m$ values were determined to be 5.88 ± 0.29

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Abbreviations: FAGLA, N-[1-(2-furyl)acryloyl]-Gly–t-Leu–NH$_2$; MOCa–PLGL(Dpa)AR, (7-methoxycoumarin-4-yl)acetyl–L-Pro–L-Leu–Gly–t-Leu–[N$^2$-(2,4-dinitrophenyl)-L-2,3-diaminopropanoyl]–t-Ala–t-Arg–NH$_2$; R-94138, N-methyl-(8S)-2-[(2R)-2-hydroxyaminocarbonylmethyl]-1-oxoundecyl[hexahydropyridazine-3-carboxamide](12), 2649–2652, 2004
and those in the presence of 10% 1-propanol were 5.80 ± 0.37 s⁻¹ and 126.8 ± 14.2 μM respectively. Upon the addition of 10% 1-propanol, Kₘ was doubled, while kₖₐ was unaltered, indicating that the inhibition of 1-propanol against matrilysin is competitive. On the basis of the competitive inhibitory manner, the Kᵢ value of 1-propanol was calculated to be 9.24% (v/v). As well as 1-propanol, all the other alcohols were shown to inhibit matrilysin in a competitive manner by Hanes–Woolf plots.

The inhibitory effects of alcohols were further examined fluorometrically by changing the alcohol concentration in the range where the reversibility of inhibition was confirmed, and the Kᵢ value was determined more precisely. For the competitive inhibition, relative activity (v/v₀) is expressed as:

\[
\frac{v}{v₀} = \frac{K_m + [S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]}
\]

where [I] is the inhibitor concentration and v₀ is the matrilysin activity in the absence of the inhibitor.

Activity decreased with increasing concentrations of 1-propanol up to 10% (Fig. 2B), and Kᵢ of 1-propanol was determined to be 8.51 ± 0.26% (v/v) or 1.14 ± 0.07 M from Eq. 1 using non-linear least-squares regression. This value is consistent with that obtained by HPLC analysis. The Kᵢ values of nine aliphatic alcohols obtained from Eq. 1 were in the range of 6.1–19.4% (v/v) or 0.66–4.80 M (Table 1). When the Kᵢ values were compared in molar concentration, the order of inhibitory potency was 2-methyl-1-propanol = 2-methyl-2-butanol > 1-butanol = 2-butanol > 1-propanol = 2-methyl-2-propanol > 2-propanol > ethanol > methanol. As for linear alcohols, the longer the carbon chain, the stronger is the inhibitory activity. When a methyl group was attached to ethanol to form 2-propanol, the
inhibitory potency was enhanced by 1.8-fold. 2-Butanol and 2-methyl-1-propanol, which have 1-propanol structures with a branched methyl group, showed 1.4 and 1.7-fold higher inhibitory potency than 1-propanol, respectively. On the other hand, 2-methyl-2-propanol and 2-methyl-2-butanol are effective in the inhibition of matrilysin, although they are not effective inhibitors against thermolysin. These observations suggest that the degree of the contribution of the S1’ subsite in the total substrate-binding affinity to thermolysin might be higher than that to matrilysin. Interestingly, 2-methyl-2-propanol and 2-methyl-2-butanol are effective in the inhibition of matrilysin, although they are not effective inhibitors against thermolysin. These observations suggest that a series of inhibitory studies with alcohols, together with structural studies such as X-ray crystallographic and NMR analyses, might be a useful method for investigating and characterizing the hydrophobic pocket at the active site of enzymes. We have reported that the interaction of matrilysin with the substrate MOCAc–PLGL(Dpa)AR and the inhibitor R-94138 is derived mainly from the hydrophobic interaction at the S1’ subsite.5,14) Accordingly, it is anticipated that introducing an appropriate group to inhibitors which enhances the hydrophobic interaction with the S1’ subsite would make them more potent and selective inhibitors against matrilysin. In the present paper, we have indicated the possibility that alcohols are suitable probes for exploring the active-site geometry of enzymes and have provided some hints for designing their inhibitors.

Acknowledgments

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References

4) Welch, A. R., Holman, C. M., Huber, M., Brenner, M. C., Browner, M. F., and Van Wart, H. E., Understanding the amino acid residue in the substrate. 2) 2-Methyl-1-propanol, which shares the structure of the Leu side chain, is the most effective inhibitor among the alcohols examined. It has been suggested that small molecules such as organic solvents bind preferentially to the active site of enzymes in spite of the availability of alternative pockets.13) Organic solvents such as 2-propanol, acetone, and acetonitrile bind primarily to the S1’ subsite of thermolysin,9) and alcohols inhibit Aeromonas proteolytica aminopeptidase by binding to its hydrophobic substrate-binding site.12) Judging by these lines of evidence, the alcohol molecules might fit into the S1’ subsite of matrilysin to exhibit the inhibition, although the possibility cannot be excluded that they bind to other subsites. It is also noted that the inhibitory effects of alcohols on matrilysin are one order of magnitude smaller than those on thermolysin (Table 1). Matrilysin, unlike thermolysin, shows no proteolytic activity toward dipeptide substrates such as FAGLA.13) Thermolysin has an active site consisting of 4 to 5 subsites, much shorter than that of matrilysin. Thus the degree of the contribution of the S1’ subsite in the total substrate-binding affinity to thermolysin might be higher than that to matrilysin.

Table 1. Inhibitor Constants (Ki) for the Inhibition of Matrilysin and Thermolysin by Alcohols

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Matrilysin</th>
<th>Thermolysin</th>
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<tbody>
<tr>
<td></td>
<td>K (µM)</td>
<td>K (µM)</td>
</tr>
<tr>
<td>Methanol (1)</td>
<td>19.4 ± 0.5%</td>
<td>1.7 ± 0.2%</td>
</tr>
<tr>
<td>Ethanol (2)</td>
<td>15.6 ± 0.4%</td>
<td>0.60 ± 0.07%</td>
</tr>
<tr>
<td>1-Propanol (3)</td>
<td>8.5 ± 0.3%</td>
<td>0.28 ± 0.04%</td>
</tr>
<tr>
<td>2-Propanol (3)</td>
<td>11.2 ± 0.3%</td>
<td>0.50 ± 0.06%</td>
</tr>
<tr>
<td>1-Butanol (4)</td>
<td>7.2 ± 0.3%</td>
<td>0.46 ± 0.06%</td>
</tr>
<tr>
<td>2-Butanol (4)</td>
<td>7.6 ± 0.3%</td>
<td>0.37 ± 0.05%</td>
</tr>
<tr>
<td>2-Methyl-1-propanol (4)</td>
<td>6.1 ± 0.3%</td>
<td>0.38 ± 0.03%</td>
</tr>
<tr>
<td>2-Methyl-2-propanol (4)</td>
<td>11.0 ± 0.4%</td>
<td>1.8 ± 0.3%</td>
</tr>
<tr>
<td>2-Methyl-2-butanol (5)</td>
<td>7.3 ± 0.2%</td>
<td>1.2 ± 0.2%</td>
</tr>
</tbody>
</table>

* Ref. 7.
* The Ki values in volume percent (%, v/v) were determined from Eq. 1.
* The Ki values in molar concentration (µM) were calculated from those in volume percent and molecular weights of alcohols.
* Numbers in parentheses indicate the number of carbon atoms in the respective alcohols.
* The Ki values are shown with the standard deviations (SD).


