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Clarification of Mechanism of Human Sputum Elastase Inhibition by a New Inhibitor, ONO-5046, Using Electrospray Ionization Mass Spectrometry

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Abstract—Liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS) to probe the nature of the covalent E–I complex was successfully applied to clarify the mechanism of human sputum elastase (HSE) inhibition by a new inhibitor, **ONO-5046**. The mass spectrum of the four HSE isozymes displayed their molecular ion peaks at $m/z = 26{,}018$, 25,929, 25,200, and 25,054, respectively. Immediately after incubation, inactivation of HSE with **ONO-5046** increased the four molecular ion peaks by approximately 84 amu, which was assigned to the mass unit of the pivaloyl moiety of **ONO-5046**. An additional minute of incubation of E–I complex restored the original molecular ion peaks. These observations strongly suggested that **ONO-5046** inactivates HSE by a reversible 'acylation–deacylation' mechanism.

Introduction

A series of pivaloyloxy benzene derivatives were discovered to be highly potent human neutrophil elastase (HNE) inhibitors.[1] Among them, *N*-{2-[4-(2,2-dimethylpropionyloxy)phenylsulfonylamino]benzoyl}aminoacetic acid (**ONO-5046**) was discovered to be a highly specific and intravenously effective neutrophil elastase inhibitor, and may be useful to study the roles of neutrophil elastase in vivo, as shown in Table 1. **ONO-5046** has been evaluated clinically with respect to a range of HNE-mediated disorders[2] and recently approved as a new drug for the treatment of acute lung injury accompanying systemic inflammatory response syndrome (SIRS) by the Ministry of Health, Labour and Welfare (MHLW) in Japan.[3]

The selective human neutrophil elastase (HNE) inhibitor, **ONO-5046**, is a mechanism-based inhibitor[4] and is thought to be transformed during the reaction with the enzyme, as illustrated in Scheme 1. The enzyme–inhibitor (E–I) complex will contain a portion of the original inhibitor. Knowledge of the component remaining in the E–I complex may provide direct evidence of the mechanism of inhibition consistent with both the chemical and kinetic data. Identification of the complexes has conventionally been performed with X-ray crystallographic analysis of the E–I complex[5] but X-ray analysis of the E–I complex consisting of **ONO-5046** and porcine pancreatic elastase (PPE) was not successful because co-crystallization of the E–I complex failed.

The recent development of electrospray ionization mass spectrometry (LC/ESI-MS) to accurately determine the mass of proteins adds a technique to resolve this kind of problem.[6,7] This technology was successfully applied to analyze the E–I complex consisting of the β-lactam inhibitor and human leukocyte elastase (HLE).[8]

From the viewpoints of both scientific and safety concerns, it is of interest to directly determine whether the proposed reversible complex is actually formed as expected during inhibition of the enzyme activity by **ONO-5046**. We analyzed the complexes produced by incubation of HSE and the newly developed **ONO-5046** by LC/ESI-MS.

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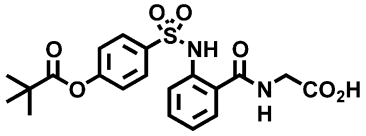
Table 1. Structure and biological activity of **ONO-5046** (**1**)



In vitro IC_{50} (μM)[a]	In vivo	
	% inhibition (iv)[b]	
	3 mg/kg	10 mg/kg
0.044	31.7 ($p<0.01$)	51.8 ($p<0.01$)

[a]IC_{50} value against HNE.
[b]Inhibitory effect of **ONO-5046** on the incease of skin capillary permeability induced by HNE in guinea pigs.

Here, we report clarification of the mechanism of inhibition of HNE by **ONO-5046** using electrospray ionization mass spectrometry.

Experimental Procedures

Materials

HSE was purchased from Elastin Products, Owensiville, MO, USA. **ONO-5046** was synthesized in our laboratory.[1]

Methods

Mass spectral analysis was performed on a Waters ZMD4000 spectrometer, equipped with an HPLC system.

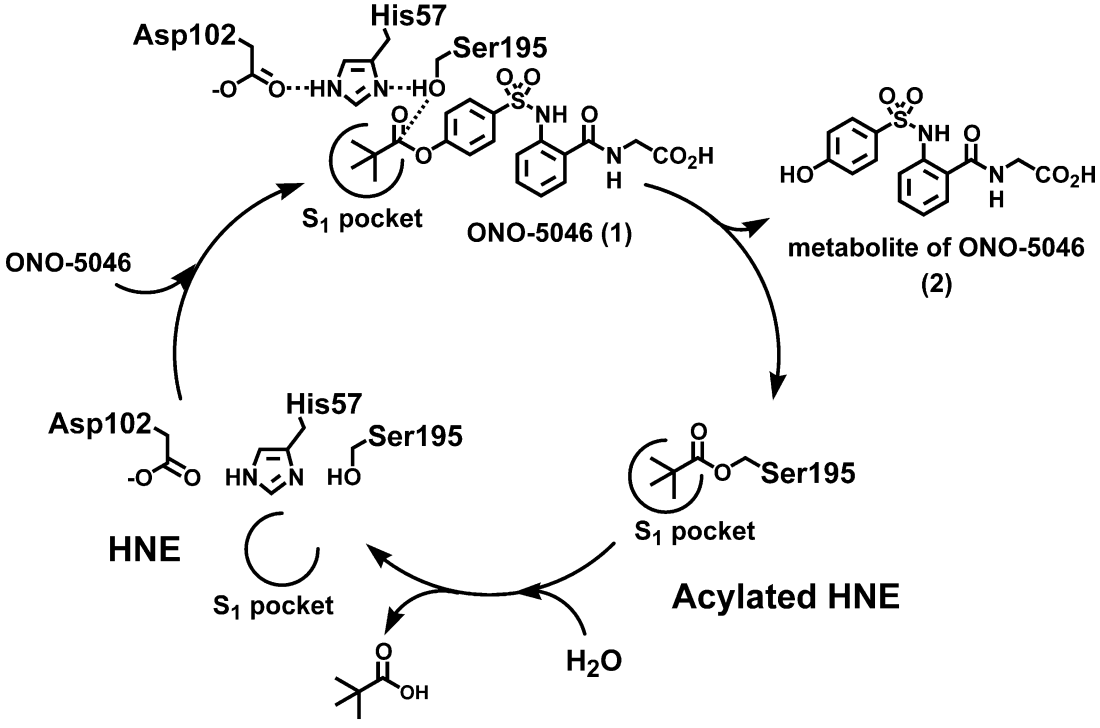
HSE (20 μmol/L) was incubated with **ONO-5046** (150 μmol/L) in HEPES (100 mmol/L) and aqueous solution of NaCl (500 mmol/L). The mixture was incubated at 37 °C for 0–20 min. After 0, 10, or 20 min of incubation at 37 °C, aliquots (5–10 μL) were injected into a Waters Symmetry 300™ loaded with a 2.1×50 mm C4 3.5 μm column and eluted with an eluent containing 0.1% acetic acid and 5–100% acetonitrile at a flow rate of 1 μL/min with gradient.

The mass data were acquired in a mass range of m/z 800–2000 with a scan duration of 2.4 s and m/z 105–500 with a scan duration of 0.5 s by turns. The cone voltage was set to 30 V. Nitrogen was used as the nebulizing and desolvation gas with flow rates of 50 and 354 L/h, respectively. Ten to 20 spectra were averaged, smoothed, and transformed using the Maximum Entropy program[9] after subtraction of a baseline.

Results and Discussion

Baugh and Travis suggested that the elastases isolated from sputum (HSE) and neutrophils (HNE) differ only in carbohydrate content.[10] Green et al. reported that HNE and HSE are catalytically identical.[11] They reported that HSE and HNE displayed identical kinetic constants such as K_m and k_{cat} toward various substrates and the inhibition constants toward cephalosporin type inhibitors are almost identical. They suggested that the carbohydrate residues of HSE and HNE are not located near the active site.

HSE is known to consist of a number of isozymes, which are catalytically indistinguishable. Determination of the molecular weight of HSE was reported in ref 4 of this article according to the results of LC/ESI-MS analysis. As illustrated in Figure 1a, four molecular ion



Scheme 1. The proposed mechanism of HNE inhibition by **ONO-5046** (**1**).

peaks ($m/z = 26{,}018$, 25,929, 25,200, and 25,054), which were assigned to the molecular weight of each isozyme, were observed according to this technique. Immediately after incubation of a mixture of HSE and **ONO-5046**, analysis of the E–I complex afforded four increased mass units ($m/z = 26{,}102$, 26,013, 25,283, and 25,138), each of which was assigned to the corresponding acylated isozyme, as indicated in Figure 1b. The observed increases in the mass units were approximately 84 amu. The increase in mass unit by adding **ONO-5046** was observed equally in synchronization among isozymes. The outcome of LC/ESI-MS analysis strongly suggested that HNE is inactivated by acylation with the pivaloyl moiety derived from **ONO-5046**. After 10 min of incubation four increased mass units ($m/z = 26{,}101$, 26,013, 25,283, and 25,138) were also observed as indicated in Figure 1c.

Another two mass units ($m/z = 435$ and 351), which were assigned to the remaining **ONO-5046** (**1**) and its possible metabolite (**2**), respectively, as described in Scheme 1, were also observed during analysis (Fig. 2). After 20 min of incubation of the mixture of HSE and **ONO-5046**, the four molecular ion peaks ($m/z = 26{,}018$, 25,930, 25,199, and 25,055), corresponding to the original isozymes, were detected again and the four newly

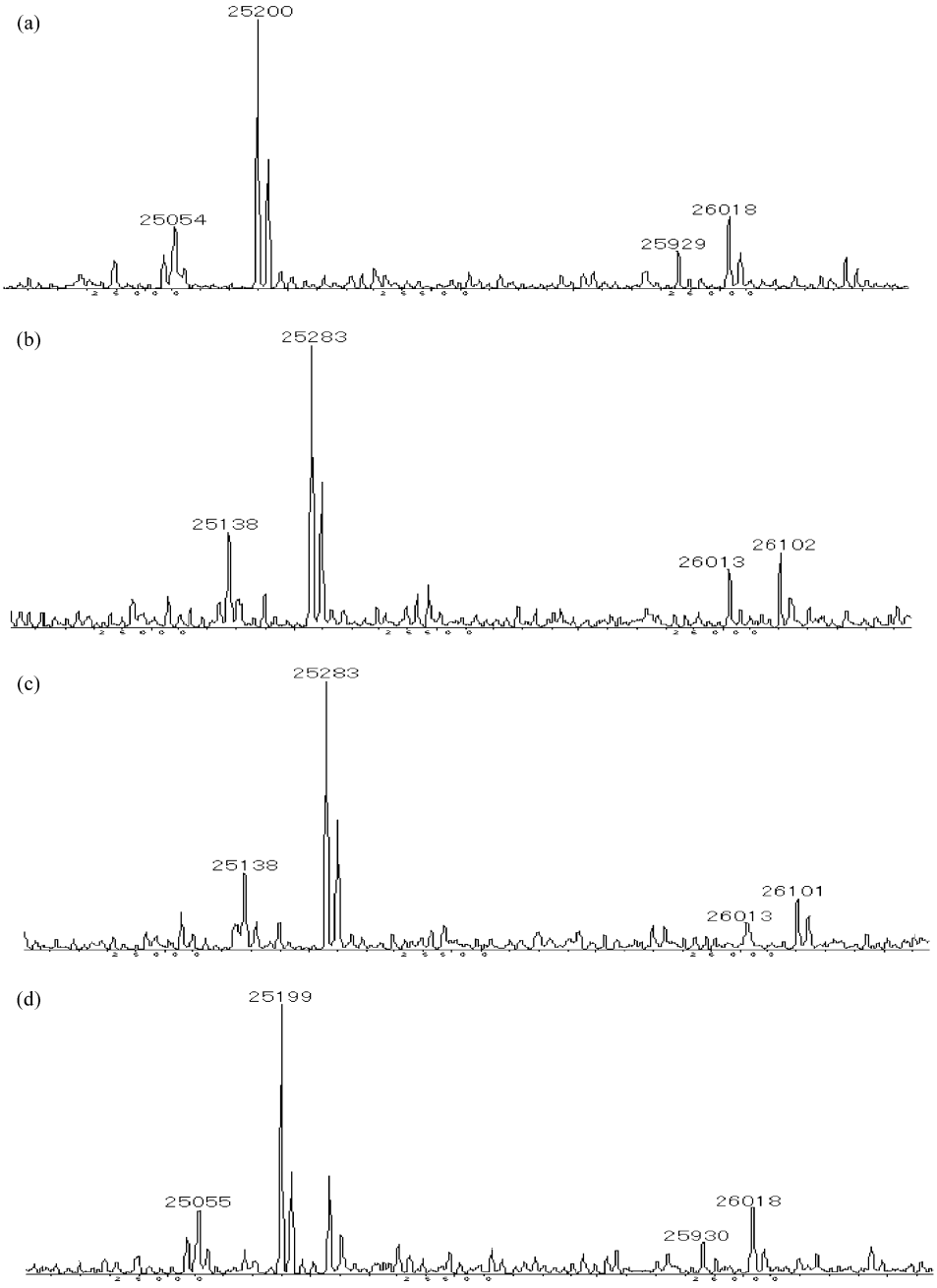


Figure 1. Positive-ion ESI mass spectra of HSE isozymes and the **ONO-5046**-derived E–I complex: (a) mass spectrum of HSE isozymes; (b) immediately after incubation of HSE and **ONO-5046** (mass spectrum of E–I complex); (c) 10 min after incubation of HSE and **ONO-5046** (mass spectrum of the E–I complex); (d) 20 min after incubation of HSE and **ONO-5046** (mass spectrum of restored HSE isozymes).

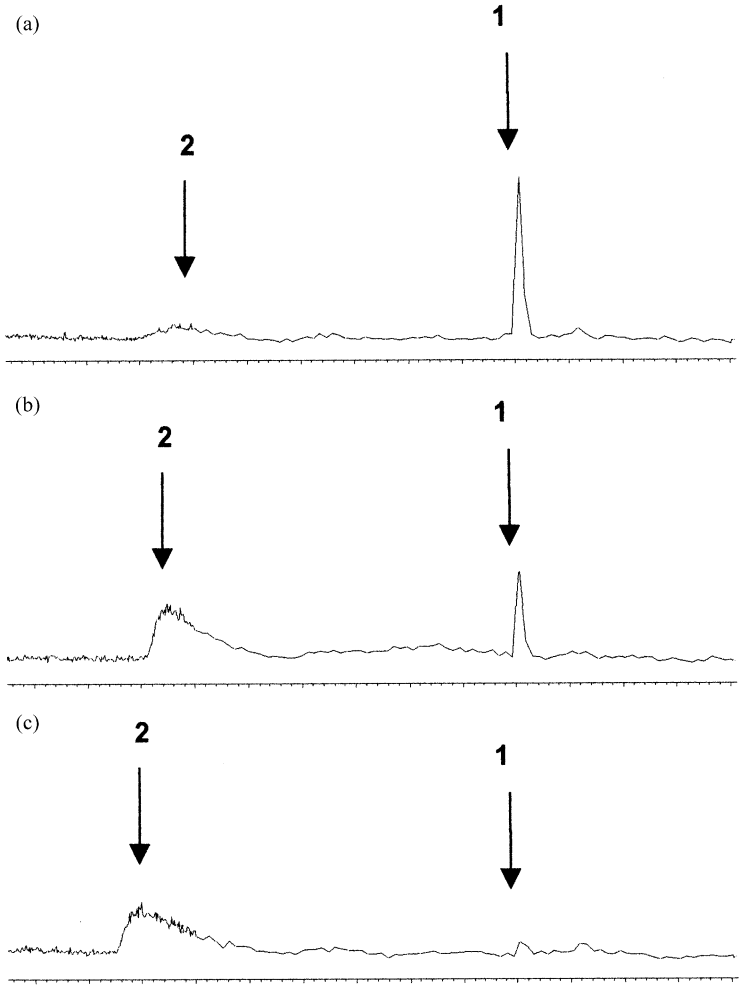


Figure 2. Total ion chromatograms acquired in a mass range of m/z 105–500; (a) immediately after incubation of HSE and **ONO-5046**: peak **1** ($m/z = 431$), which was assigned to **ONO-5046** (**1**), was observed; (b) 10 min after incubation of HSE and **ONO-5046**: peak **2** ($m/z = 351$), which was assigned to a possible metabolite (**2**), was observed; (c) 20 min after incubation of HSE and **ONO-5046**: peak **1** ($m/z = 435$) was no longer detected.

observed mass units ($m/z = 26{,}101$, 26,013, 25,283, and 25,138) and the molecular ion peak of **ONO-5046** ($m/z = 435$) were no longer detected (Figs. 1d and 2c). This result clearly demonstrated that the original HSE was restored by hydrolysis of the E–I complex.

Based on the experimental results described above, the mechanism of HSE inhibition by the new inhibitor **ONO-5046** appeared to proceed through acylation of the enzyme, as described in Scheme 1. **ONO-5046** was estimated to show hydrophobic interactions between its pivaloyl group and Val 216 of the S1 site of elastase. Thus, the reversible 'acylation–deacylation mechanism' of **ONO-5046** is one plausible explanation for the fewer safety problems compared to the irreversible elastase inhibitors reported to date.

Conclusion

In summary, liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS) was used to examine the mechanism of inhibition of the new HNE inhibitor, **ONO-5046**, which has recently been approved by the MHLW as a new drug for the treatment of acute lung injury accompanying SIRS. According to the results of analysis of the E–I complex after 0–10 min of incubation using this method, acylation of the enzyme by the pivaloyl moiety derived from **ONO-5046** was detected as an increase of approximately 84 amu. This increase in the mass units was not detected after 20 min of incubation. The reversible mechanism of inhibition of **ONO-5046** was attributed to one of the suggested explanations for its safety relative to the irreversible inhibitors reported to date.

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