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# Direct interaction of ONO-5046 with human neutrophil elastase through $^1$H NMR and molecular docking

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ABSTRACT

Human neutrophil elastase (HNE) has been implicated as a major contributor in the pathogenesis of diseases, such as pulmonary emphysema, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and other inflammatory diseases. Therefore, searching for appropriate and potential human neutrophil elastase inhibitors (HNEI) that would restore the balance between the free enzyme and the endogenous inhibitors would be of therapeutic interest. ONO-5046 is the first specific HNEI to improve respiratory function and protect lung tissues against various lung injuries. However, the mechanism of ONO-5046 to HNE is still unclear. In this study, the binding properties of ONO-5046 were investigated through $^1$H NMR, molecular docking, and bioassay methods to understand the effect of ONO-5046 to HNE. The proton spin–lattice relaxation rate and molecular rotational correlation time results indicated that ONO-5046 has higher affinity with HNE. The molecular docking study showed that ONO-5046 is perfectly matched for the primary enzyme specificity pocket (S1 pocket), and is tightly bound to this pocket of HNE through hydrophobic and hydrogen bonding interactions. The results of both methods were validated through analysis of the HNE inhibitory activity bioassay of ONO-5046 with an $IC_{50}$ value of 87.05 nM. Our data suggested that ONO-5046 could bind to HNE through direct interaction, and that molecular docking and NMR methods are valid approaches to survey new HNEI.



## 1. Introduction

Human neutrophil elastase (HNE) is a major serine proteinase in the azurophilic granules of neutrophils and one of the destructive enzymes with optimal activity around neutral pH. HNE release in the physiological defense system could be controlled by endogenous inhibitors. However, intense neutrophil infiltration leads to an imbalance between the amount of HNE and endogenous inhibitors in some pathological conditions [1–3]. The superabundant HNE causes the degradation of the tissue matrix and the deterioration of inflammation in various disease states, including pulmonary emphysema, cystic fibrosis, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and other inflammatory processes. Therefore, the importance of HNE as a pathogenic agent in various disease processes has aroused interest in searching for appropriate and potential human neutrophil elastase inhibitors (HNEI) [4,5].

The activities of endogenous inhibitors have been suggested to be circumvented because of high molecular weight or sensitivity to oxygen radicals [6,7]. Under these circumstances, several low-weight synthetic HNE inhibitors (HNEI) have been studied as therapeutic agents. ONO-5046 (N-[2-[4-(2,2-dimethyl-propionyloxy) phenyl-sulfonyl-amino]benzoyl]aminoacetic acid) (Fig. 1), a specific low-weight HNEI, has been approved in Japan for the treatment of ALI/ARDS with systemic inflammatory response syndrome (SIRS) [8]. ONO-5046 is not sensitive to active oxygen radicals and effectively penetrates to the lesion location to improve pulmonary function in patients through attenuating the influx and stiffness of neutrophils and the permeability of pulmonary vessels [9,10]; reducing the levels of some inflammatory mediators, such as NO, interleukin 6, interleukin 8, and TNF-α, among others [11–13]. However, ONO-5046 does not improve the mortality outcome of ALI/ARDS patients [14]. Kawabata [15] mentioned that ONO-5046 could directly inhibit the activity of HNE through simple bioassay and that the blockade of HNE activity would be a useful therapeutic strategy for lung injuries; however, the direct action mode still needs further study.

In the present study, the direct combination mode of ONO-5046 with HNE was investigated using NMR spectroscopy, molecular docking, and HNE inhibitory activity bioassay methods to evaluate the validity of NMR spectroscopy and molecular docking methods for the new HNEI, which is currently screened from natural products.

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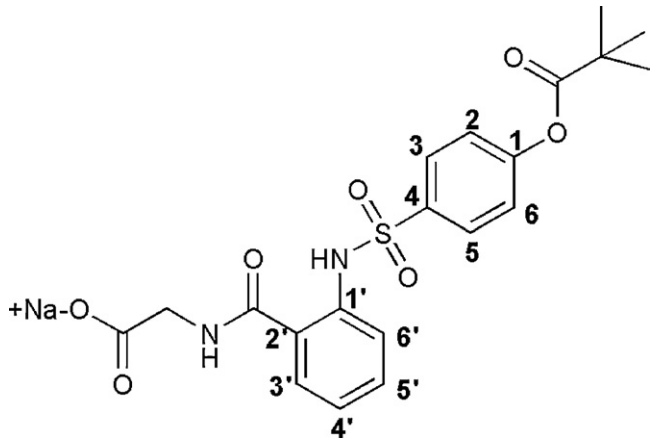


**Fig. 1.** Chemical Structure of ONO-5046.

## 2. Materials and methods

### 2.1. Materials

HNE (EC 3.4.21.37), from human leukocytes was purchased from Innovative Research Company (Novi, Michigan). ONO-5046, HNE substrate (MeO-Suc-Ala-Ala-Pro-Val-pNA), and soybean trypsin inhibitor was obtained from Sigma (St. Louis, USA) and used without further purification. All other reagents were of analytical grade.

### 2.2. NMR spectroscopy

The sample for NMR study was prepared in 99.9% $D_2O$ buffered at pH 7.0 (50 mM phosphate buffer) containing 1% $d_6$-DMSO. The pH value was carefully adjusted to 7.0 with either DCl or NaOD. The final concentrations of ONO-5046 and HNE were 1.0 mM and 5.0 μM, respectively. All measurements were performed on a Bruker Avance 400 MHz NMR spectrometer at 298 K. Suppression of the residual water signal was achieved through the Watergate pulse program with gradients.

$^1$H NMR spectra were recorded using a BBO broadband probe. A total of 64 scans were collected into 4 K data points at a spectral width of 4000 Hz. The spin–lattice relaxation rates were measured using the $(180^\circ–\tau–90^\circ–t)_n$ sequence. The $\tau$ values used for the selective and nonselective experiments were as follows: 0.00001, 0.001, 0.01, 0.1, 0.2, 0.4, 0.6, 0.7, 0.8, 1, 1.2, 1.5, 2, 3, and 5.0 s, respectively, and the delay time ($t$) was 0.5 s. The 180° selective inversion of the proton spin population was obtained via a selective Gaus1_180i.1000 shape pulse with a length of 20 ms and a power of 50 dB, corresponding to an excitation width of about 45 Hz [16]. All relaxation rates were calculated in the initial rate approximation [17]. All NMR data were processed using the Bruker Topspin 2.1 software.

### 2.3. Molecular docking

The crystal structure (PDB ID: 1B0F) of HNE in complex with the inhibitor SEI (1-{3-methyl-2-[4-(morpholine-4-carbonyl)-benzoylamino]-butyryl}-pyrrolidine-2-carboxylic acid (3,3,4,4,4-pentafluoro-1-isopropyl-2-oxo-butyl)-amide) was downloaded from the RCSB protein data bank [18]. The potential of the 3D structure of HNE was assigned according to the Amber force field with Kollman-United-atom charges encoded in the molecular modeling software Sybyl 7.3 (Linux-os2x). The essential hydrogen atoms of HNE were distributed through Sybyl 7.3. The initial structure of ONO-5046 was recovered from the PubChem Compound home (http://www.ncbi.nlm.nih.gov/pccompound). The geometry of ONO-5046 was simply optimized by applying the Gasteiger-Marsili-atom charges.

The advanced docking program Autodock 3.0.3 was used to perform the automated molecular docking for handling the interaction mode of ONO-5046 with HNE [19,20]. The Lamarckian genetic algorithm (LGA) described the relationship between the inhibitors and enzymes through orientation, translation, and conformation of the inhibitors. The number of energy evaluations, population size, generations, and docking runs were set to 250,000, 50, 27,000, and 20, respectively, based on the requirement of the Amber force field. The root mean square deviation (RMSD) between the conformational superposition of SEI from the X-ray crystal structure and that from the AutoDock result was 0.44 Å, indicating that the parameter set for the AutoDock simulation was reasonable to reproduce the X-ray structure. In addition, the AutoDock method and the parameters set could be extended to search for the binding conformations of other inhibitors accordingly. ONO-5046 of the training set was manually docked into the SEI binding pocket (S1 pocket) of the above-mentioned HNE enzyme. Each docking cycle consisted of a fitness evaluation, cross-over, mutation, and selection. A Solis and Wets local search implemented the energy minimization on a user-specified proportion of population [21].

Finally, the docked complexes of the inhibitor-enzyme were selected using the criteria of interacting energy combined with geometrical matching quality. Subsequently, 40 docked conformations of the two ligands were analyzed in terms of energy, hydrogen bonding, and hydrophobic interaction between the ligand and the protein. The conformation corresponding to the lower binding energy and the key amino acid residues in HNE interaction with ligand was selected as the best binding conformation.

### 2.4. HNE activity inhibitory bioassay

The HNE inhibitory activity of ONO-5046 was evaluated using a previously described procedure [22]. Briefly, 200 μl substrate solution (1.4 mM MeO-Suc-Ala-Ala-Pro-Val-pNA in Tris–HCl buffer, 10 mM, pH 7.5) was mixed with different concentration gradients of ONO-5046 solution (stock solutions of ONO-5046 were dissolved in dimethyl sulfoxide and diluted with Tris–HCl buffer to give the final sample concentrations). Up to 3 μl enzyme solution (0.03 U HNE) was added, and then the mixture was incubated for 1 h at 37 °C in the dark. Afterward, the reaction was quenched by adding 200 μl soybean trypsin inhibitor at a concentration of 0.2 mg/ml. The absorbance was then immediately measured at 405 nm using a Plate reader (Bio-Tek). The group which the substrate was replaced with 10 mM pH 7.5 Tris–HCl buffer solutions was used as a zero alignment of ONO-5046. The inhibitory activity of ONO-5046 was decided using the following equations:

$$\text{Inhibitory ratio} = \frac{[A_{\text{control}} - (A_{\text{sample}} - A_{\text{zero}})]}{A_{\text{control}}} \times 100\%$$

$A_{\text{control}}$: the absorbance of the group with only HNE and the substrate, $A_{\text{sample}}$: the absorbance of the group with ONO-5046, the substrate, and HNE, $A_{\text{zero}}$: the absorbance of the group with ONO-5046, Tris–HCl buffer solution, and HNE.

The absorbance data expressed as mean ± standard deviation were obtained from three separate experiments. Statistical analysis of the data was performed using one-way ANOVA followed by Dunnett-$t$ test. A $p$ value < 0.05 was considered statistically significant.