

1 **Title:** Neutrophil elastase inhibitor sivelestat ameliorates gefitinib-naphthalene-induced
2 acute pneumonitis in mice

3 **Running Title:** Sivelestat resolves pneumonitis

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20 **Key words:** Acute pneumonitis; gefitinib; neutrophil elastase inhibitor

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22 **Summary statement:** Neutrophil elastase inhibitor sivelestat is a promising therapeutic
23 agent for severe acute pneumonitis caused by gefitinib.

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34 **ABSTRACT:**

35 Background and objective: Gefitinib, an epidermal growth factor receptor-tyrosine kinase
36 inhibitor (EGFR-TKI), is an effective therapeutic agent for non-small cell lung cancer with
37 EGFR mutations. It can cause severe acute pneumonitis in some patients. We previously
38 demonstrated that mice with naphthalene-induced airway epithelial injury developed
39 severe gefitinib-induced pneumonitis and that neutrophils played important roles in the
40 development of the disease. This study aimed to investigate the effects of the neutrophil
41 elastase inhibitor sivelestat on gefitinib-induced pneumonitis in mice.

42 Methods: C57BL/6J mice received naphthalene (200 mg/kg) intraperitoneally on day 0.
43 Gefitinib (250 or 300 mg/kg) was orally administered to mice from day -1 until day 13.
44 Sivelestat (150 mg/kg) was administered intraperitoneally from day 1 until day 13.

45 Bronchoalveolar lavage fluid (BALF) and lung tissues were sampled on day 14.

46 Results: Sivelestat treatment significantly reduced the protein level, neutrophil count,
47 neutrophil elastase activity in BALF, and severity of histopathologic findings on day 14
48 for mice administered with 250 mg/kg of gefitinib. Moreover, sivelestat treatment
49 significantly improved the survival of mice administered with 300 mg/kg of gefitinib.

50 Conclusions: These results indicate that sivelestat is a promising therapeutic agent for
51 severe acute pneumonitis caused by gefitinib.

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67 **INTRODUCTION:**

68 Gefitinib, an epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), is an
69 effective therapeutic agent for non-small cell lung cancer with EGFR mutations (Harada et
70 al., 2011; Mok et al., 2009). It can cause severe acute pneumonitis in some patients.

71 Characteristics of patients who developed interstitial pneumonia included old age, poor
72 performance status, a history of smoking, and preexisting interstitial pneumonia (Harada et
73 al., 2011; Kudoh et al., 2008).

74 Injuries to respiratory epithelium and alveolar epithelial cells are regarded as the initial
75 phenomena of various respiratory illnesses, such as acute respiratory distress syndrome,
76 interstitial pneumonia, and chronic obstructive pulmonary disease.

77 Neutrophil elastase is a protease produced by neutrophils. Excessive neutrophil elastase
78 can cause lung tissue damage by direct cytotoxicity to endothelial and epithelial cells and
79 by degradation of key structural elements of connective tissue, such as elastin, collagen,
80 and proteoglycan (Yamada et al., 2011; Lee and Downey, 2001).

81 Sivelestat, a small molecule (529 Da), is a neutrophil elastase inhibitor developed and
82 produced by Ono Pharmaceutical Company in Japan (Aikawa et al., 2011). In the animal
83 models of acute lung injury (ALI), the beneficial effects of sivelestat have been reported in
84 bleomycin-induced inflammation and streptococcus pneumonia (Yuan et al., 2014;
85 Yamada et al., 2011). A phase 3 study in Japan demonstrated that sivelestat improved the
86 investigator assessment of pulmonary function and significantly reduced the duration of
87 intensive care required for patients with ALI associated with systemic inflammatory
88 response syndrome (SIRS) (Tamakuma et al., 2004). In 2002, sivelestat was approved in
89 Japan for the treatment of ALI associated with SIRS (Aikawa et al., 2011). After approval,
90 a phase 4 study indicated that it contributed to early weaning from mechanical ventilation
91 (Aikawa et al., 2011). The beneficial effects of sivelestat have also been reported in several
92 other models, including lipopolysaccharide (LPS)-induced lung inflammation, ozone-
93 induced airway response, and bleomycin-induced pulmonary fibrosis (Yuan et al., 2014;
94 Matsumoto et al., 1999; Takemasa et al., 2012).

95 Pulmonary stem cells are important for tissue recovery. The club cell is a type of
96 pulmonary stem cell found in the distal airway. Stem cell abnormality is considered to
97 promote chronic lung injury and lung fibrosis (Harada et al., 2011; Gazdhar et al., 2007).
98 Naphthalene has club cell-selective cytotoxicity (Harada et al., 2011; Van Winkle et al.,
99 1995; Stripp et al., 1995). Therefore, we previously used a naphthalene-induced lung

100 injury model as an animal model containing a risk factor of gefitinib-induced pneumonia.
101 We found that gefitinib administration after naphthalene treatment prolonged ALI with
102 neutrophil infiltration on day 14. Laser capture microdissection and microarray analysis of
103 the terminal bronchial epithelial cells showed upregulation of the genes included—*S100a8*,
104 *S100a6*, *Stfa3*, *Trim23*, and *Mug1*, which are known to participate in inflammatory cell
105 chemotaxis, activation, and migration (Harada et al., 2011; Raquil et al., 2008; Ozato et al.,
106 2008). Although the precise mechanisms involved remain unclear, gefitinib treatment
107 prolonged lung inflammation by upregulating neutrophil chemoattractant genes from
108 peripheral epithelial cells. Therefore, we hypothesize that sivelestat plays a protective role
109 against gefitinib-induced lung injury by inhibiting neutrophilic inflammation.

110

111 **RESULTS:**

112 **Sivelestat improved the survival rate of gefitinib-induced pneumonitis in mice**

113 The administration of 300-mg/kg gefitinib with 150-mg/kg sivelestat following
114 naphthalene significantly improved the survival rate on day 14 compared with that of
115 300 mg/kg gefitinib following naphthalene (Figure 2).

116

117 **Sivelestat ameliorated the loss of body weight of gefitinib-induced pneumonitis in mice**

118 **mice**
119 We measured mice body weight to determine the general influence of ALI. Weight loss is
120 a good marker for the severity of naphthalene-induced lung injury (Harada et al., 2011;
121 Verschoyle et al., 1997). On day 7, the body weights of mice treated with naphthalene
122 alone were significantly decreased; however, by day 14, the body weights returned to the
123 level of the control injected with corn oil. On day 14, body weights of mice treated with
124 250 mg/kg gefitinib following naphthalene remained significantly decreased compared
125 with that of mice treated with naphthalene alone, whereas the body weights of mice treated
126 with 250 mg/kg gefitinib and 150 mg/kg sivelestat following naphthalene were
127 significantly increased compared with that of mice treated with 250 mg/kg gefitinib
128 following naphthalene (Figure 3).

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130 **Sivelestat ameliorated the lung inflammation of gefitinib-induced pneumonitis in mice**

131 **mice**
132 Histopathological examination

133 We used histologic cell analysis to examine the degree of inflammatory cell infiltration.
134 Naphthalene alone induced neutrophil infiltration on day 7 but not on day 14 in the lung
135 tissue, as previously described (Harada et al., 2011). The administration of 250 mg/kg
136 gefitinib following naphthalene aggravated neutrophil infiltration and induced alveolar
137 hemorrhage on day 14 (Figure 4A). In contrast, the administration of 250 mg/kg gefitinib
138 with 150 mg/kg sivelestat following naphthalene significantly decreased the pathologic
139 grade compared with that of 250 mg/kg gefitinib following naphthalene (Figure 4B).
140 BALF analysis
141 On day 14, the number of neutrophils, total cell count, and protein concentration in BALF
142 of mice treated with 250 mg/kg gefitinib following naphthalene were significantly
143 increased compared with those of mice treated with naphthalene alone. On day 14, the
144 administration of 250 mg/kg gefitinib and 150 mg/kg sivelestat following naphthalene
145 significantly decreased the number of neutrophils, total cell count, and protein
146 concentration in BALF compared with that with 250 mg/kg gefitinib following
147 naphthalene (Figure 5).
148 In addition, the administration of 250 mg/kg gefitinib and 150 mg/kg sivelestat following
149 naphthalene significantly decreased the level of IL-8 (Figure 6A) and neutrophil elastase
150 activity (Figure 6B) in BALF compared with those resulting from the administration of
151 250 mg/kg gefitinib following naphthalene.

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153 **DISCUSSION**

154 As we reported previously, the presumed sequence of the mechanism of gefitinib–
155 naphthalene pneumonitis is as follows: 1) the upregulation of neutrophil chemoattractant
156 genes in bronchiolar epithelial cells; 2) neutrophil migration into alveolar space and
157 interstitial tissues, and; 3) release of neutrophil elastase from neutrophils, resulting in lung
158 tissue damage.

159 IL-8 is produced by alveolar epithelial cell line (A549), airway epithelial cells, and
160 inflammatory cells, such as macrophages and neutrophils. IL-8 is a well-known
161 neutrophilic chemoattractant. Some studies have reported that gefitinib induces the
162 production of IL-8 from alveolar epithelial cell line (A549). Neutrophil elastase induces
163 the release of IL-8 from bronchial epithelial cells (Yamada et al., 2011; Nakamura et al.,
164 1992), which in turn recruits additional neutrophils. Our study indicated that
165 administration of sivelestat decreased neutrophil elastase, which in turn inhibited the level

166 of IL-8 in gefitinib–naphthalene-induced pneumonitis. In other words, sivelestat treatment
167 could halt the negative spiral of lung injury. A limitation of the current study was that we
168 could not identify the IL-8 producing cells targeted by sivelestat; therefore, further studies
169 are required.

170 A significant reduction in the number of club cells has been observed in the airway
171 epithelium of chronic tobacco smokers (Nomori et al., 1994). The naphthalene-induced
172 club cell injury in a mice model may be representative of patients at a high risk of
173 gefitinib-induced pneumonitis. This suggests that the presence of peripheral airway
174 damage may increase the susceptibility of patients with lung cancer to interstitial
175 pneumonia during treatment with gefitinib.

176 In conclusion, we demonstrated a treatment strategy for ALI caused by gefitinib. Currently,
177 new generation EGFR-TKIs, such as afatinib and osimertinib, are used for treating non-
178 small cell lung cancer with EGFR mutations. It is well known that the new generation
179 EGFR-TKIs also induce pneumonitis; therefore, the current study should be repeated for
180 the new generation EFGR-TKIs.

181

182 **MATERIALS AND METHODS:**

183 Animal treatment

184 The experiments were approved by the Committee on Ethics Regarding Animal
185 Experiments of Kyushu University. C57BL/6 female mice (7 weeks old; SLC, Inc,
186 Shizuoka Japan) were used in all experiments.

187 Naphthalene (Wako Pure Chemical Industries, Osaka, Japan) was injected
188 intraperitoneally on day 0 (200 mg/kg). Gefitinib (Caymann Chemical, Arizona, USA)
189 stirred into 1% Tween 80 (Wako) was daily administered orally on days –1 to 13. We
190 administrated gefitinib at two doses: 1) 250 mg/kg as a tolerated dose and 2) 300 mg/day
191 as the 50% lethal dose (LC₅₀). The neutrophil elastase inhibitor sivelestat (Ono
192 Pharmaceutical, Osaka, Japan) in saline was daily injected intraperitoneally on days 1 to
193 13 (150 mg/kg). A scheme of the administration schedule is shown in Figure 1.

194

195 Histopathological evaluation

196 Histopathology was performed as previously described (Harada et al., 2011; Hamada et al.,
197 2008).

198 The right lung was fixed in 10% buffered formalin and embedded in paraffin, and the lung
199 sections were stained with hematoxylin and eosin. The pathological grade of inflammation
200 in the whole area of the midsagittal was evaluated under $\times 200$ magnification and
201 determined according to the following criteria: 0 = no lung abnormality; 1 = presence of
202 inflammation involving $<25\%$ of the lung parenchyma; 2 = lesions involving 25–50% of
203 the lung; and 3 = lesions involving $>50\%$ of the lung.

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205 Bronchoalveolar lavage

206 The bronchoalveolar lavage (BAL) method and analysis was performed as previously
207 described (Harada et al., 2011; Hamada et al., 2008). After counting the cell numbers in
208 BAL fluid (BALF), cells were cytospun and stained with Diff-Quick for classification. The
209 BALF supernatant was freeze-dried using a lyophilizer. The lyophilized samples were
210 dissolved to determine total protein concentrations, interleukin-8 (IL-8), and neutrophil
211 elastase activity. Total protein concentrations in BALF were measured using the Bio-Rad
212 Protein Assay.

213

214 ELISA for assessment of IL-8 in the BALF

215 The concentration of IL-8 in BALF was determined using mouse cytokine ELISA kits
216 (R&D Systems, Minneapolis, MN, USA).

217

218 Neutrophil elastase activity

219 Neutrophil elastase activity in BALF was determined using the highly neutrophil elastase-
220 specific synthetic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide. Briefly,
221 samples were incubated in 0.1-M Tris-HCl buffer (pH 8.0) containing 0.5-M NaCl and 1-
222 mM substrate for 24 h at 37 °C. After incubation, *p*-nitroaniline was measured
223 spectrophotometrically at 405 nm, considered to be a measure of neutrophil elastase
224 activity (Hagio et al., 2004; Yanagihara et al., 2007).

225

226 Statistical Analysis

227 The Student's t-test was used for the comparison of body weight, number of BALF cells,
228 protein concentration, IL-8 concentration, neutrophil elastase activity, histopathological
229 grade, and survival curves. $P < 0.05$ was considered significant. Statistical analysis was
230 performed in the statistical software package JMP version 11 (SAS Institute, Cary, NC).

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233 Ltd. for the provision of sivelestat as the study medication.

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235 **COMPETING INTERESTS:**

236 The authors declare no competing or financial interests

237

238 **AUTHOR CONTRIBUTIONS:**

239 All authors designed research. H.M. and S.-O. S. performed research. T.Y. , N.H., E. H.,
240 C.-I.H., M.-A.O., K. S., T. Y., and T.N. provided assistance. H.M. and S.-O. S. analyzed
241 data. H.M. and T. Y. wrote the paper. All authors approved the final manuscript.

242

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314

315 **FIGURE LEGENDS:**

316 **Figure 1.** Experimental scheme detailing the administration of naphthalene, gefitinib, and
317 sivelestat. po: oral administration, ip: intraperitoneal administration

318 **Figure 2.** Kaplan–Meier survival curve

319 Survival study of mice with the administration of 300 mg/kg gefitinib and 150 mg/kg
320 sivelestat following naphthalene improved the survival rate compared with that of
321 300 mg/kg gefitinib following naphthalene ($n = 10$). * $P < 0.05$.

322 **Figure 3.** Changes in mice body weight over time.

323 Body weights of mice treated with naphthalene alone were significantly decreased on
324 day 7; however, by day 14, the body weights returned to the level of the control. On day 14,
325 the body weights of mice treated with 250 mg/kg gefitinib following naphthalene remained
326 significantly decreased compared with that of mice treated with naphthalene alone,
327 whereas the body weights of mice treated with 250 mg/kg gefitinib and 150 mg/kg

328 sivelestat following naphthalene were significantly increased compared with those of mice
329 at treated with 250 mg/kg gefitinib following naphthalene.

330 Compared with control group at the same point ($n = 7$). $*P < 0.05$, $**P < 0.01$.

331 **Figure 4.** Histologic assessment of lung tissues on day 14. (A) Hematoxylin and eosin
332 staining. The administration of 250 mg/kg gefitinib following naphthalene significantly
333 induced neutrophil infiltration and acute lung injury. The administration of 250 mg/kg
334 gefitinib and 150 mg/kg sivelestat following naphthalene improved neutrophil infiltration
335 compared with that with 250 mg/kg gefitinib following naphthalene ($n = 5$), Scale bars:
336 100 μm . (B) Pathologic grade of lung tissues on day 14 ($n = 5$). $**P < 0.01$.

337 **Figure 5.** Bronchoalveolar lavage fluid (BALF) analysis on day 14

338 (A) The administration of 250 mg/kg gefitinib following naphthalene significantly induced
339 the upregulation of total cell count and neutrophil recruitment, which were decreased by
340 administration of 250 mg/kg gefitinib and 150 mg/kg sivelestat following naphthalene
341 ($n = 5$). $**P < 0.01$.

342 (B) The administration of 250 mg/kg gefitinib following naphthalene significantly induced
343 the upregulation of protein concentration, which was decreased by the administration of
344 250 mg/kg gefitinib and 150 mg/kg sivelestat following naphthalene ($n = 5$). $**P < 0.01$.

345 **Figure 6.** Interleukin-8 (IL-8) and neutrophil elastase activity in bronchoalveolar lavage
346 fluid (BALF) on day 14

347 (A) IL-8 was increased by the administration of 250 mg/kg gefitinib following naphthalene
348 and was decreased by administration of 250 mg/kg gefitinib and 150 mg/kg sivelestat
349 following naphthalene ($n = 5$). $*P < 0.05$.

350 (B) Neutrophil elastase was increased by the administration of 250 mg/kg gefitinib
351 following naphthalene and was decreased by administration of 250 mg/kg gefitinib and
352 150 mg/kg sivelestat following naphthalene ($n = 5$). $**P < 0.01$.











