

Axl and immune checkpoints inhibitors from fruiting bodies of *Pleurocybella porrigens*

| | |
|------------------------------|--|
| 著者 | Ridwan Arif Yanuar, Wu Jing, Harada Etsuko, D' Alessandro-Gabazza Corina N., Toda Masaaki, Yasuma Taro, Gabazza Esteban C., Choi Jae-Hoon, Hirai Hirofumi, Kawagishi Hirokazu |
| journal or publication title | The Journal of Antibiotics |
| volume | 73 |
| page range | 733-736 |
| year | 2020-05-27 |
| 出版者 | Springer Nature |
| 権利 | This is a post-peer-review, pre-copyedit version of an article published in The journal of antibiotics. The final authenticated version is available online at: https://doi.org/10.1038/s41429-020-0323-4 |
| URL | http://hdl.handle.net/10297/00027863 |

doi: 10.1038/s41429-020-0323-4

1 Brief Communication

2 **Axl and immune checkpoints inhibitors from fruiting bodies**
3 **of *Pleurocybella porrigens***

4 Arif Yanuar Ridwan ¹ • Jing Wu ² • Etsuko Harada ³ • Corina N. D`Alessandro-Gabazza ⁴ •
5 Masaaki Toda ⁴ • Taro Yasuma ⁴ • Esteban C. Gabazza ⁴ • Jae-Hoon Choi ² • Hirofumi Hirai
6 ^{1,2} • Hirokazu Kawagishi ^{1,2,*}

7 ¹ Graduate School of Science and Technology, Shizuoka University, Shizuoka Japan

8 ² Research Institute of Green Science and Technology, Shizuoka University, Shizuoka, Japan

9 ³ Department of Forest and Environmental Science, Miyazaki University, Miyazaki, Japan

10 ⁴ Department of Immunology, Graduate School of Medicine, Mie University, Mie, Japan

11

12 * Corresponding author:

13 Hirokazu Kawagishi

14 kawagishi.hirokazu@shizuoka.ac.jp

15

16

17 **Abstract**

18 A novel compound (**1**) and three known ones (**2–4**) were isolated from the fruiting bodies of
19 *Pleurocybella porrigens*. The structure of the novel compound was determined by 1D and 2D
20 NMR and HRESIMS data. The biological activity of **1–3** was evaluated using the A549 lung
21 cancer cell line. The results showed the inhibitory activity of compounds **1–3** on the expression
22 of Axl and immune checkpoint molecules.

23 **Keywords:** butenolide derivatives/ lung cancer cell inhibitor/ *Pleurocybella porrigens*/ structure determination

24

25

26

27

28

29

30

31

32

33 In 2004, there was a poisoning outbreak affecting 55 Japanese people after eating the wild
34 mushroom *Pleurocybella porrigens* (Sugihiratake in Japanese). Among them, seventeen people
35 died due to acute encephalopathy. This outbreak has led to several investigations to clarify the
36 mechanism of mushroom poisoning. In recent years, our group has reported the isolation and
37 characterization of a novel lectin and unusual amino acids from *P. porrigens* [1,2]. Our subsequent
38 investigation led to the discovery of pleurocybellaaziridine, a structurally-unique and unstable
39 amino acid as the candidate of toxic principle [3]. Studies by other groups also suggested that the
40 poisoning outbreak might have been caused by vitamin D analogue, cyanide and thiocyanate [4,5].
41 Despite the highly toxic nature of *P. porrigens*, only a few studies on metabolites with
42 physiological activity have been so far reported. Apart from elucidating the molecular mechanism
43 of the disease caused by the fungus, we are also interested in search for bioactive secondary
44 metabolites produced by *P. porrigens*, because the fungus produces structurally unique compounds
45 such as pleurocybellaaziridine. Recently, we are focusing on search for Axl and immune
46 checkpoints (PD-L1/PD-L2) inhibitors from natural sources, especially mushroom-forming fungi.

47 Based on this background, we attempted to isolate bioactive metabolites to evaluate their
48 activity. In the present study, we report the isolation of a new butenolide (**1**) along with three
49 known compounds (**2–4**), their inhibitory activity against Axl and immune checkpoint molecules
50 using the A549 lung cancer cell line.

51 The fresh fruiting bodies of *P. porrigens* (24 kg) were collected at Narusawa village,
52 Yamanashi Prefecture, in Japan. The fruiting bodies were crushed and extracted with ethanol
53 (EtOH) and then acetone. The solutions were combined, concentrated under reduced pressure, and
54 divided into *n*-hexane, ethyl acetate (EtOAc), ethanol, and water-soluble parts. The EtOAc soluble
55 part (72.7 g) was subjected to silica gel column chromatography {CH₂Cl₂/acetone = 100:0, 90:10,
56 80:20, 70:30, 60:40 (v/v); CH₂Cl₂/MeOH = 90:10, 70:30, 60:40, 50:50, 0:100 (v/v)} to obtain
57 thirteen fractions (fractions 1–13). Fraction 7 (1.0 g) was further separated by ODS column
58 chromatography (70%, 80%, 90% MeOH, MeOH), affording ten fractions (fractions 7–1 to 7–10).
59 Compound **1** (10.2 mg) was isolated from fraction 7–2 by reverse-phase HPLC (Capcell Pak C18
60 AQ, 40% MeCN). Fraction 5 was separated by MPLC, Silica 60Å, 40 g, *n*-hexane: EtOAc= 85:15
61 (v/v) to yield seven fractions (fractions 5–1 to 5–7), and fraction 5–4 was further separated by
62 reverse-phase HPLC (Capcell Pak C18 AQ, 70% MeCN) to obtain compound **2** (2.2 mg). Fraction
63 8 was separated by MPLC, Silica 60Å, 40 g, *n*-hexane: acetone = 60:40 (v/v) to afford nine

64 fractions (fractions 8–1 to 8–9), and fraction 8–5 was eluted by 60% MeOH using Sep-Pak ODS.
65 The 60% MeOH elution part of fraction 8–5 was further fractionated by reverse-phase HPLC
66 (Cosmosil Cholester, 40% MeOH) to yield 14 fractions (fractions 8–5–1 to 8–5–14). Compound
67 **3** (3.0 mg) was isolated from fraction 8–5–5 by reverse-phase HPLC (Cosmosil PBr, 40% MeOH).
68 Fraction 6 (12 g) was separated using MPLC, Silica 60Å, 40 g, *n*-hexane: EtOAc = 60:40 (v/v) to
69 obtain 7 fractions (fractions 6–1 to 6–7). Fraction 6–4 was further fractionated by MPLC (ODS,
70 120Å, 37 g, 70% MeOH) to give 11 fractions (fractions 6–4–1 to 6–4–11) and then fraction 6–4–3
71 was separated by reverse-phase HPLC (Cosmosil PBr, 70% MeCN) to afford compound **4** (1.3
72 mg).

73 Compound **1** was isolated as a pale yellow oil, and its molecular formula was established as
74 C₁₅H₂₄O₅ by HRESIMS *m/z* 307.1510 [M+Na]⁺ (calcd for 307.1521, C₁₅H₂₄NaO₅), indicating
75 four degrees of unsaturation. Based on ¹H-, ¹³C-NMR and DEPT data, compound **1** showed the
76 presence of two singlet methyls, eight methylenes, and five quarternary carbons. The complete
77 assignment of all the protons and carbons was accomplished as shown in Table 1. In the HMBC
78 spectrum, a singlet methyl group, 4'-CH₃ (δ_H 1.75) showed correlations to C-5' (δ_C 172.7), C-4'
79 (δ_C 125.0), C-3' (δ_C 158.2), and 3'-CH₃ (δ_H 1.88) showed correlations to C-4' (δ_C 125.0), C-3' (δ_C
80 158.2), and C-2' (δ_C 107.4), resulting the construction of the structure of a dimethyl butenolide. In
81 addition, the HMBC correlations between H-2 (δ_H 2.28) to C-4 (δ_C 29.2), C-3 (δ_C 24.6), C-1 (δ_C
82 179.2) and H-3 (δ_H 1.58) to C-4 (δ_C 29.2), C-2 (δ_C 34.0) and C-1 (δ_C 179.2) indicated a carboxyl
83 group with an alkyl chain (Fig. 1b). Based on the 1D and 2D NMR as well as the molecular formula
84 of the compound, the planar structure of **1** was established as shown (Fig. 1a). Previously, a
85 structurally similar compound with **1** has been reported. The compound possesses a methoxy and
86 a methoxycarbonyl groups instead of the hydroxy and carboxy groups in **1**, however, its absolute
87 configuration has not determined yet [6]. In order to determine the absolute configuration of **1**, its
88 specific rotation and CD data were compared to those of compound **2** that was isolated in this
89 study: **1**, [α]_D²⁵ + 4.65 (*c* = 1.02, MeOH), CD data {λ_{max} nm (Δε): 269 (-3.93), 335 (-0.34); **2**,
90 [α]_D²⁷ - 15 (*c* = 0.22, MeOH), CD λ_{max} nm (Δε): 269 (+3.32), 336 (+0.01) (Fig. 1a and S1). The
91 absolute configuration of **2** has been determined already [7,8]. The specific rotation of **1** was also
92 similar to that of a known compound, sinularone I, which possesses a longer chain and a terminal

93 ethyl ester group $\{[\alpha]^{25}_D + 5.4 (c = 0.18, \text{MeOH})\}$ [8]. The result allowed us to conclude the
94 absolute configuration of **1** to be *S*.

95 Compound **2** $\{(R)5\text{-hydroxy-3,4-dimethyl-5-pentylfuran-2}(5H)\text{-one}\}$ has been isolated from
96 the endophytic fungus *Epichloe typhina*, however, to our knowledge, this was the first isolation
97 from mushroom-forming fungi [9]. Interestingly, the absolute configurations of **1** and **2** were
98 opposite to each other. Both of the compounds are a kind of lactol. There might be equilibrium
99 between the lactol form and ring-opening form, resulting in the epimerization at C-2'. The similar
100 case has been reported; homaxinolides A and B from the marine sponge *Homaxinella* sp. have
101 opposite configurations to each other [10].

102 Compound **3**, cyclo(L-Leu-D-Pro), has been isolated from the marine sponge *Stelletta clavosa*
103 and *Bacillus* sp. as antimicrobial compound [11,12]. Compound **4** (9-ethoxy-9-oxononanoic acid)
104 has been isolated from the endophytic fungus *E. typhina* and exhibited antifungal activity [9].

105 Axl, a receptor tyrosine kinase, and programmed death ligands 1 (PD-L1) and 2 (PD-L2) have
106 been clinically reported as promising targets in cancer treatment [13,14]. Overexpression of Axl
107 is correlated with the progression of several cancers such as glioblastoma multiforme, breast, and
108 lung cancer [15–17]. Axl plays an important role in the epithelial-mesenchymal transition (EMT),
109 which is an important step for the initiation of metastasis and development of resistance to drug
110 and chemotherapy [18,19]. On the other hand, programmed cell death-1 (PD-1) is an important
111 inhibitory receptor expressed on the surface of activated T cells and B cells that is activated after
112 binding to its ligands PD-L1 and PD-L2 [20,21]. Receptor activation leads to suppression and
113 death of T cells, and therefore the expression of PD-L1 and PD-L2 by cancer cells is an important
114 mechanism contributing to cancer cell immune escape [14]. The expression of Axl, and PD-L1
115 and PD-L2 may not be regulated by the same mechanism, however, a recent study found the
116 correlation between Axl and immune checkpoint molecules in lung adenocarcinomas; Axl
117 positively contributes to the expression of immune checkpoints in the regulation of immune
118 microenvironment and tumor proliferation [17]. Therefore, if the expression of these molecules
119 was downregulated by a small compound, the compound might become a promising candidate as
120 an anti-cancer reagent.

121

122

123 In this work, we assessed the effect of compounds 1–3 on the expression of Axl, PD-L1, and
124 PD-L2 using the A549 lung cancer line. The results showed that compounds 1–3 significantly
125 inhibit the expression of Axl, PD-L1, and PD-L2. Among them, the suppressive activity of
126 compound 3 was the most potent against Axl and PD-L1 (Fig. 2). These findings suggest the
127 potential compounds 1–3 for using as therapy to block cancer immune escape mediated by
128 checkpoint molecules and Axl. There is no much study showing the presence Axl and immune
129 checkpoint inhibitors in natural products. Recently, our group has reported the isolation of
130 compounds that reduce the expression of Axl and immune checkpoint molecules from the edible
131 mushroom *Leucopaxillus giganteus* [22]. Our findings indicate that mushroom is a potential source
132 of natural Axl and PD-L1/PD-L2 inhibitors with the potential therapeutic application.

133
134 **Acknowledgements** This research was funded by a Grant-in Aid for Scientific Research on
135 Innovative Areas “Frontier Research on Chemical Communications” from MEXT (No
136 117H06402) and Specific Research Grant from Takeda Science Foundation.

137
138 **Compliance with ethical standards**

139
140 **Conflict of interest** The authors declare that they have no conflict of interest.

141
142 **Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in
143 published maps and institutional affiliations.

144 Supplementary information is available at (The Journal of Antibiotics) website

145
146 **References**

- 147 1. Kawaguchi T, Suzuki T, Kobayashi Y, Kodani S, Hirai H, Kawagishi H, et al. Unusual amino acid derivatives
148 from the mushroom *Pleurocybella porrigens*. *Tetrahedron*. 2010;66(2):504–7.
- 149 2. Suzuki T, Amano Y, Fujita M, Kobayashi Y, Dohra H, Kawagishi H, et al. Purification, characterization, and
150 cDNA cloning of a lectin from the mushroom *Pleurocybella porrigens*. *Biosci Biotechnol Biochem*.
151 2009;73(3):702–9.
- 152 3. Wakimoto T, Asakawa T, Akahoshi S, Suzuki T, Nagai K, Kawagishi H, et al. Proof of the existence of an
153 unstable amino acid: Pleurocybellaziridine in *Pleurocybella porrigens*. *Angew Chemie - Int Ed*.
154 2011;50(5):1168–70.
- 155 4. Sasaki H. Sugihiratake mushroom (Angel’s Wing Mushroom)-induced cryptogenic encephalopathy may
156 involve vitamin D analogues. *Biol Pharm Bull*. 2006;29(12):2514–8.
- 157 5. Akiyama H, Toida T, Sakai S, Amakura Y, Kondo K, Sugita-Konishi Y, et al. Determination of cyanide and
158 thiocyanate in Sugihiratake mushroom using HPLC method with fluorometric detection. *J Heal Sci*.
159 2006;52(1):73–7.

- 160 6. Nomura Y, Kusumi T, Ishitsuka M, Kakisawa H. 2,3-dimethyl-4-methoxybutenolides from red algae,
161 *Coeloseira pacifica* and *Ahnfeltia paradoxa*. Chem Lett. 1980;277:955–6.
- 162 7. Shi H, Yu S, Liu D, Van Ofwegen L, Proksch P, Lin W. Sinularones A-I, new cyclopentenone and butenolide
163 derivatives from a marine soft coral *Simularia* sp. and their antifouling activity. Mar Drugs. 2012;10(6):1331–
164 44.
- 165 8. Zhang J, Liang Y, Liao XJ, Deng Z, Xu SH. Isolation of a new butenolide from the South China Sea gorgonian
166 coral *Subergorgia suberosa*. Nat Prod Res. 2014;28(3):150–5.
- 167 9. Koshino H, Yoshihara T, Sakamura S, Shimanuki T, Sato T, Tajimi A. Novel C-11 Epoxy Fatty Acid from
168 *Stromata* of *Epichloe typhina* on *Phleum pratense*. Agric Biol Chem. 1989;53(9):2527–8.
- 169 10. Mansoor TA, Hong J, Lee CO, Sim CJ, Im KS, Lee DS, et al. New cytotoxic metabolites from a marine sponge
170 *Homaxinella* sp. J Nat Prod. 2004;67(4):721–4.
- 171 11. Wegerski CJ, France D, Cornell-Kennon S, Crews P. Using a kinase screen to investigate the constituents of
172 the sponge *Stelletta clavosa* obtained from diverse habitats. Bioorganic Med Chem. 2004;12(21):5631–7.
- 173 12. Kumar N, Mohandas C, Nambisan B, Kumar DRS, Lankalapalli RS. Isolation of proline-based cyclic
174 dipeptides from *Bacillus* sp. N strain associated with rhabditid entomopathogenic nematode and its
175 antimicrobial properties. World J Microbiol Biotechnol. 2013;29(2):355–64.
- 176 13. Rankin EB, Giaccia AJ. The receptor tyrosine kinase Axl in cancer progression. Cancers (Basel). 2016;8(11).
- 177 14. Azuma K, Ota K, Kawahara A, Hattori S, Iwama E, Harada T, et al. Association of PD-L1 overexpression
178 with activating EGFR mutations in surgically resected nonsmall-cell lung cancer. Ann Oncol.
179 2014;25(10):1935–40.
- 180 15. Hutterer M, Knyazev P, Abate A, Reschke M, Maier H, Stefanova N, et al. Axl and growth arrest-specific
181 gene 6 are frequently overexpressed in human gliomas and predict poor prognosis in patients with
182 glioblastoma multiforme. Clin Cancer Res. 2008;14(1):130–8.
- 183 16. Wang X, Saso H, Iwamoto T, Xia W, Gong Y, Pusztai L, et al. TIG1 promotes the development and
184 progression of inflammatory breast cancer through activation of Axl kinase. Cancer Res. 2013;73(21):6516–
185 25.
- 186 17. Tsukita Y, Fujino N, Miyauchi E, Saito R, Fujishima F, Itakura K, et al. Axl kinase drives immune checkpoint
187 and chemokine signalling pathways in lung adenocarcinomas. Mol Cancer. 2019;18(1):1–6.
- 188 18. He R, Wang B, Wakimoto T, Wang M, Zhu L, Abe I. Cyclodipeptides from metagenomic library of a Japanese
189 marine sponge. J Braz Chem Soc. 2013;24(12):1926–32.
- 190 19. Zhu C, Wei Y, Wei X. Axl receptor tyrosine kinase as a promising anti-cancer approach: Functions, molecular
191 mechanisms and clinical applications. Mol Cancer. 2019;18(1).
- 192 20. Tang S, Kim PS. A high-affinity human PD-1/PD-L2 complex informs avenues for small-molecule immune
193 checkpoint drug discovery. Proc Natl Acad Sci U S A. 2019;116(49):24500–6.
- 194 21. Zak KM, Grudnik P, Magiera K, Dömling A, Dubin G, Holak TA. Structural Biology of the Immune
195 Checkpoint Receptor PD-1 and Its Ligands PD-L1/PD-L2. Structure. 2017;25(8):1163–74.
- 196 22. Malya IY, Wu J, Harada E, Toda M, D'Alessandro-Gabazza CN, Kawagishi H, et al. Plant growth regulators
197 and Axl and immune checkpoint inhibitors from the edible mushroom *Leucopaxillus giganteus*. Biosci
198 Biotechnol Biochem. 2020. <https://doi.org/10.1080/09168451.2020.1743170>.

199
200
201
202

203 Titles and legends to figures

204 Fig. 1 **a** Chemical structures of compounds **1–4**. **b** Key ^1H – ^1H COSY and ^1H – ^{13}C HMBC
205 correlations of compound **1**

206 Fig. 2 Effect of compounds **1–3** Axl, PD-L1, and PD-L2 on lung cancer cells A549. Values
207 indicate means with standard deviation from three independent triplicate experiments. Statistical
208 analysis was performed using Fisher's test (** $P < 0.05$ vs control, $n = 3$)

209

210

211

212

Table 1 ^1H and ^{13}C NMR data of compound **1** in CDCl_3 .

| Position | δ_{H} ($J = \text{Hz}$) | δ_{C} |
|--------------------|---|---------------------|
| 1 | - | 179.2 |
| 2 | 2.28 (t, $J = 7.3$) | 34.0 |
| 3 | 1.58 (m) | 24.6 |
| 4 | 1.25 (m) | 29.2 |
| 5 | 1.25 (m) | 29.0 |
| 6 | 1.25 (m) | 28.9 |
| 7 | 1.25 (m) | 28.8 |
| 8 | 1.06 (m), 1.22 (m) | 22.8 |
| 9 | 1.73 (m), 1.92 (m) | 35.8 |
| 2' | - | 107.4 |
| 3' | - | 158.2 |
| 4' | - | 125.0 |
| 5' | - | 172.7 |
| 3'-CH ₃ | 1.88 (s) | 10.7 |
| 4'-CH ₃ | 1.75 (s) | 8.3 |

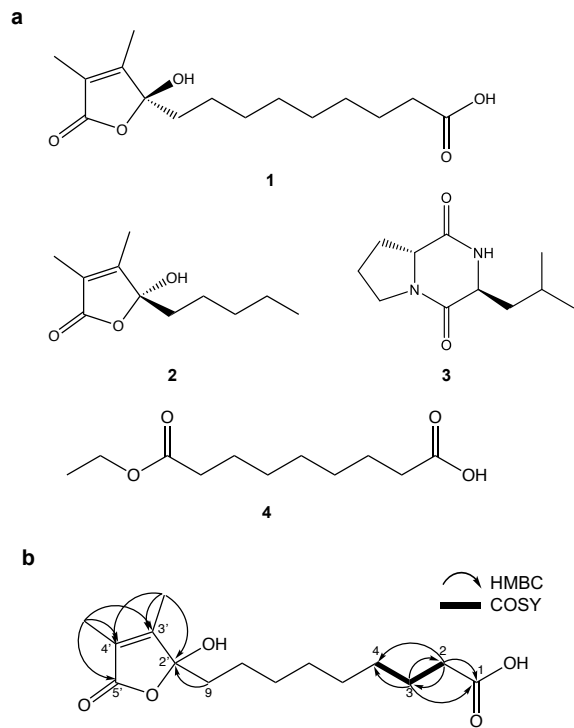
213

214

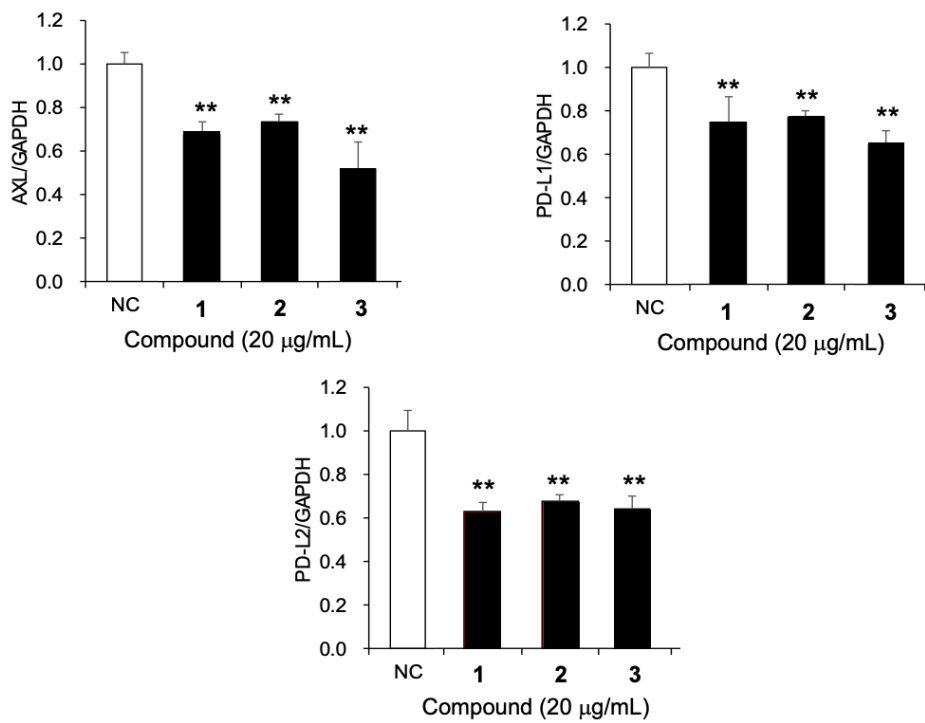
The ^{13}C and ^1H NMR were measured at 125 and 500 MHz, respectively

215

216



217
218 Figure 1
219



220
221 Figure 2