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Brief Communication

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

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Abstract

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

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

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 {CH_2Cl_2/acetone = 100:0, 90:10,
56 80:20, 70:30, 60:40 (v/v); CH_2Cl_2/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

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

Compound **1** was isolated as a pale yellow oil, and its molecular formula was established as $C_{15}H_{24}O_5$ by HRESIMS *m/z* 307.1510 $[M+Na]^+$ (calcd for 307.1521, $C_{15}H_{24}NaO_5$,), indicating four degrees of unsaturation. Based on ^{1}H-, ^{13}C-NMR and DEPT data, compound **1** showed the presence of two singlet methyls, eight methylenes, and five quarternary carbons. The complete assignment of all the protons and carbons was accomplished as shown in Table 1. In the HMBC spectrum, a singlet methyl group, 4'-CH_3 (δ_H 1.75) showed correlations to C-5' (δ_C 172.7), C-4' (δ_C 125.0), C-3' (δ_C 158.2), and 3'-CH_3 (δ_H 1.88) showed correlations to C-4' (δ_C 125.0), C-3' (δ_C 158.2), and C-2' (δ_C 107.4), resulting the construction of the structure of a dimethyl butenolide. In 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 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 group with an alkyl chain (Fig. 1b). Based on the 1D and 2D NMR as well as the molecular formula of the compound, the planar structure of **1** was established as shown (Fig. 1a). Previously, a structurally similar compound with **1** has been reported. The compound possesses a methoxy and a methoxycarbonyl groups instead of the hydroxy and carboxy groups in **1**, however, its absolute configuration has not determined yet [6]. In order to determine the absolute configuration of **1**, its specific rotation and CD data were compared to those of compound **2** that was isolated in this study: **1**, $[\alpha]^{25}_D$ + 4.65 (*c* = 1.02, MeOH), CD data {λ_{max} nm ($\boldsymbol{\Delta\varepsilon}$): 269 (-3.93), 335 (-0.34); **2**, $[\alpha]^{27}_D$ - 15 (*c* = 0.22, MeOH), CD λ_{max} nm ($\boldsymbol{\Delta\varepsilon}$): 269 (+3.32), 336 (+0.01) (Fig. 1a and S1). The absolute configuration of **2** has been determined already [7,8]. The specific rotation of **1** was also 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, MeOH)}[8]. The result allowed us to conclude the
94 absolute configuration of **1** to be *S*.

95 Compound **2** {(*R*)5-hydroxy-3,4-dimethyl-5-pentylfuran-2(5*H*)-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

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

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Compliance with ethical standards

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

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203 Titles and legends to figures

204 Fig. 1 **a** Chemical structures of compounds **1**–**4**. **b** Key $^{1}H–^{1}H$ COSY and $^{1}H–^{13}C$ 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, $n = 3$)

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

Position	δ_H (J = Hz)	δc
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 The ^{13}C and ^{1}H NMR were measured at 125 and 500 MHz, respectively

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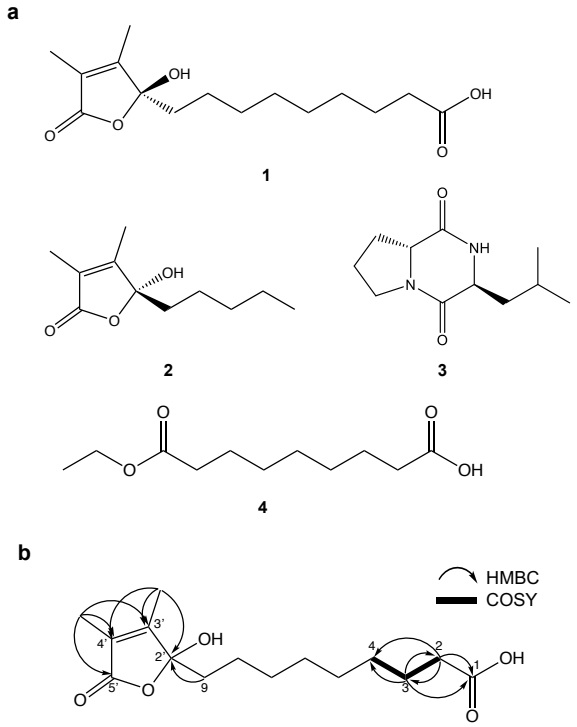

Figure 1

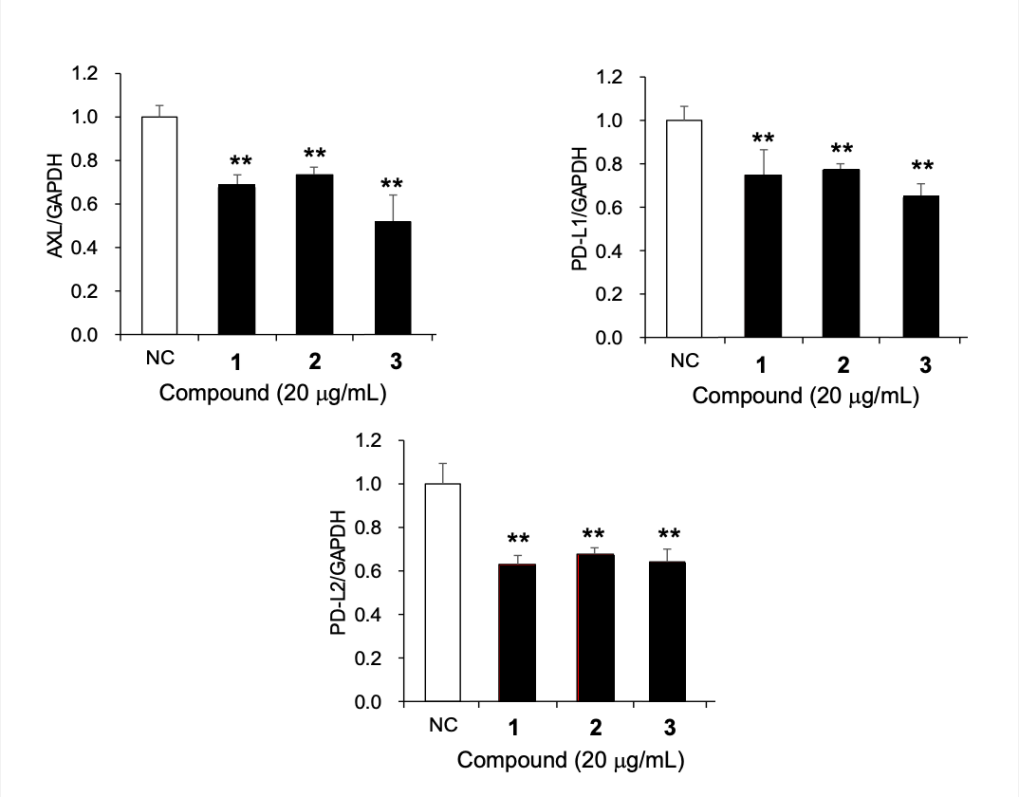

Figure 2