Axl and immune checkpoints inhibitors from fruiting bodies of Pleurocybella porrigens

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

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

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

The fresh fruiting bodies of *P. porrigens* (24 kg) were collected at Narusawa village, Yamanashi Prefecture, in Japan. The fruiting bodies were crushed and extracted with ethanol (EtOH) and then acetone. The solutions were combined, concentrated under reduced pressure, and divided into *n*-hexane, ethyl acetate (EtOAc), ethanol, and water-soluble parts. The EtOAc soluble part (72.7 g) was subjected to silica gel column chromatography {CH_2Cl_2/acetone = 100:0, 90:10, 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 thirteen fractions (fractions 1–13). Fraction 7 (1.0 g) was further separated by ODS column chromatography (70%, 80%, 90% MeOH, MeOH), affording ten fractions (fractions 7–1 to 7–10). Compound **1** (10.2 mg) was isolated from fraction 7–2 by reverse-phase HPLC (Capcell Pak C18 AQ, 40% MeCN). Fraction 5 was separated by MPLC, Silica 60Å, 40 g, *n*-hexane: EtOAc= 85:15 (v/v) to yield seven fractions (fractions 5–1 to 5–7), and fraction 5–4 was further separated by reverse-phase HPLC (Capcell Pak C18 AQ, 70% MeCN) to obtain compound **2** (2.2 mg). Fraction 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 ($\Delta\varepsilon$): 269 (-3.93), 335 (-0.34); **2**, $[\alpha]^{27}_D$ - 15 (*c* = 0.22, MeOH), CD λ_{max} nm ($\Delta\varepsilon$): 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

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

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

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

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

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.

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

References

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

6. Nomura Y, Kusumi T, Ishitsuka M, Kakisawa H. 2,3-dimethyl-4-methoxybutenolides from red algae, *Coeloseira pacifica* and *Ahnfeltia paradoxa*. Chem Lett. 1980;277:955–6.

7. Shi H, Yu S, Liu D, Van Ofwegen L, Proksch P, Lin W. Sinularones A-I, new cyclopentenone and butenolide derivatives from a marine soft coral *Sinularia* sp. and their antifouling activity. Mar Drugs. 2012;10(6):1331–44.

8. Zhang J, Liang Y, Liao XJ, Deng Z, Xu SH. Isolation of a new butenolide from the South China Sea gorgonian coral *Subergorgia suberosa*. Nat Prod Res. 2014;28(3):150–5.

9. Koshino H, Yoshihara T, Sakamura S, Shimanuki T, Sato T, Tajimi A. Novel C-11 Epoxy Fatty Acid from *Stromata* of *Epichloe typhina* on *Phleum pratense*. Agric Biol Chem. 1989;53(9):2527–8.

10. Mansoor TA, Hong J, Lee CO, Sim CJ, Im KS, Lee DS, et al. New cytotoxic metabolites from a marine sponge *Homaxinella* sp. J Nat Prod. 2004;67(4):721–4.

11. Wegerski CJ, France D, Cornell-Kennon S, Crews P. Using a kinase screen to investigate the constituents of the sponge *Stelletta clavosa* obtained from diverse habitats. Bioorganic Med Chem. 2004;12(21):5631–7.

12. Kumar N, Mohandas C, Nambisan B, Kumar DRS, Lankalapalli RS. Isolation of proline-based cyclic dipeptides from *Bacillus* sp. N strain associated with rhabitid entomopathogenic nematode and its antimicrobial properties. World J Microbiol Biotechnol. 2013;29(2):355–64.

13. Rankin EB, Giaccia AJ. The receptor tyrosine kinase Axl in cancer progression. Cancers (Basel). 2016;8(11).

14. Azuma K, Ota K, Kawahara A, Hattori S, Iwama E, Harada T, et al. Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer. Ann Oncol. 2014;25(10):1935–40.

15. Hutterer M, Knyazev P, Abate A, Reschke M, Maier H, Stefanova N, et al. Axl and growth arrest-specific gene 6 are frequently overexpressed in human gliomas and predict poor prognosis in patients with glioblastoma multiforme. Clin Cancer Res. 2008;14(1):130–8.

16. Wang X, Saso H, Iwamoto T, Xia W, Gong Y, Pusztai L, et al. TIG1 promotes the development and progression of inflammatory breast cancer through activation of Axl kinase. Cancer Res. 2013;73(21):6516–25.

17. Tsukita Y, Fujino N, Miyauchi E, Saito R, Fujishima F, Itakura K, et al. Axl kinase drives immune checkpoint and chemokine signalling pathways in lung adenocarcinomas. Mol Cancer. 2019;18(1):1–6.

18. He R, Wang B, Wakimoto T, Wang M, Zhu L, Abe I. Cyclodipeptides from metagenomic library of a Japanese marine sponge. J Braz Chem Soc. 2013;24(12):1926–32.

19. Zhu C, Wei Y, Wei X. Axl receptor tyrosine kinase as a promising anti-cancer approach: Functions, molecular mechanisms and clinical applications. Mol Cancer. 2019;18(1).

20. Tang S, Kim PS. A high-affinity human PD-1/PD-L2 complex informs avenues for small-molecule immune checkpoint drug discovery. Proc Natl Acad Sci U S A. 2019;116(49):24500–6.

21. Zak KM, Grudnik P, Magiera K, Dömling A, Dubin G, Holak TA. Structural Biology of the Immune Checkpoint Receptor PD-1 and Its Ligands PD-L1/PD-L2. Structure. 2017;25(8):1163–74.

22. Malya IY, Wu J, Harada E, Toda M, D'Alessandro-Gabazza CN, Kawagishi H, et al. Plant growth regulators and Axl and immune checkpoint inhibitors from the edible mushroom *Leucopaxillus giganteus*. Biosci Biotechnol Biochem. 2020. https://doi.org/10.1080/09168451.2020.1743170.

Titles and legends to figures

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

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

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

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

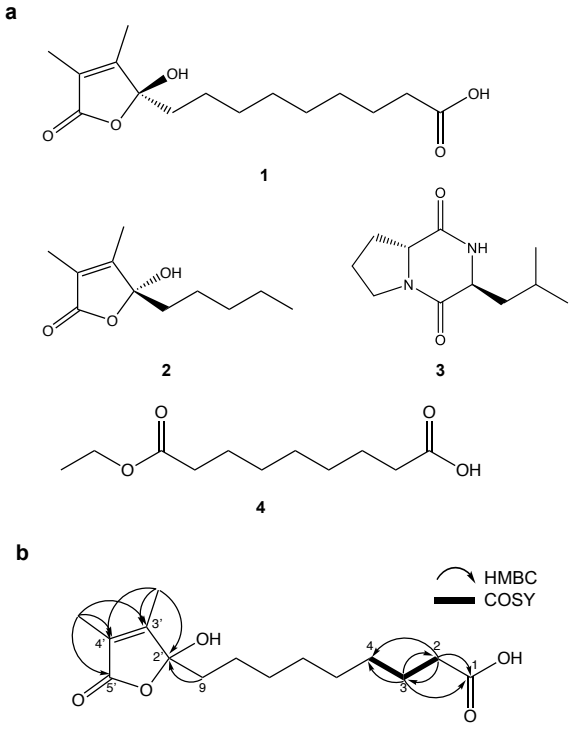

Figure 1

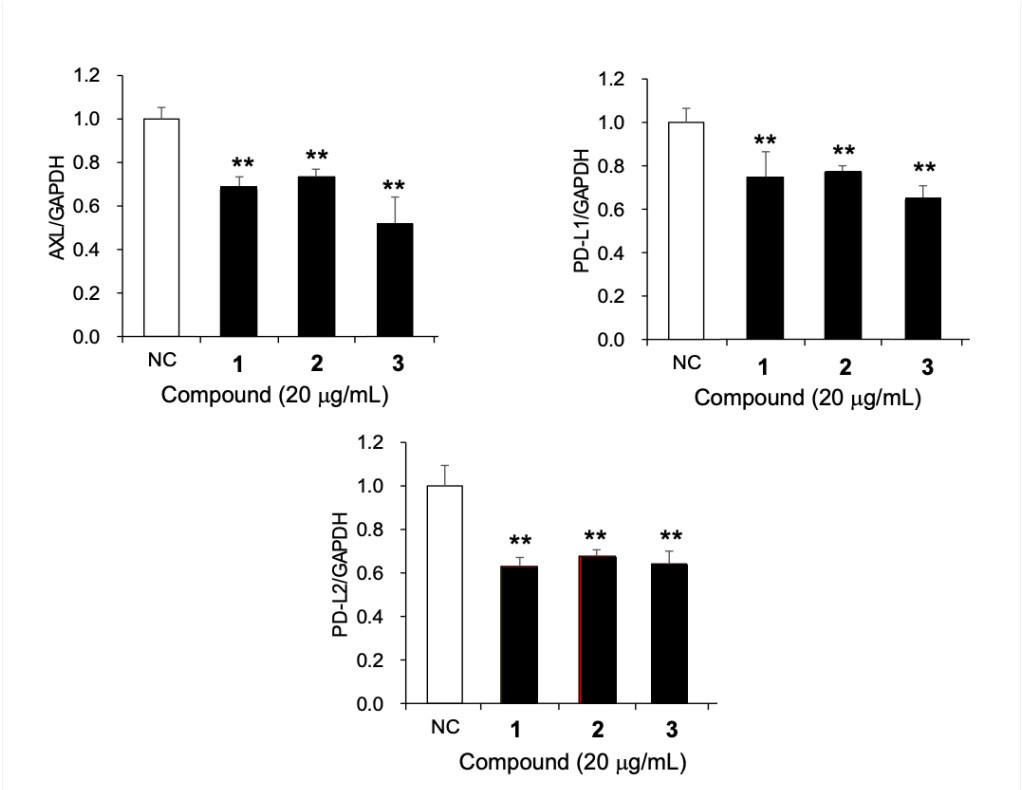

Figure 2