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NOTE

A cell-based screening to detect inhibitors of BRAF signaling pathway

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BRAF, a serine/threonine kinase, regulates the mitogen-activated protein kinase (RAS/RAF/MEK/ERK) pathway, which is involved in the transduction of mitogenic signals from the cell membrane to the nucleus. BRAF is known to be mutated in 70% of malignant melanomas. The most common BRAF mutation is V600E, and it increases the kinase activity that constitutively stimulates ERK signaling, cell proliferation and survival. The mutation is an early event for the development of melanoma and it is present in 80% of primary melanomas.[1,2] Recently, it has been reported that mutant BRAF protein is a client of Hsp90, which is required for the folding and stability of mutant BRAF. Therefore, BRAF-mutated cells are more sensitive to the Hsp90 inhibitor, compared with BRAF wild-type cells.[3] On the basis of the above-mentioned background, we established the screening system using BRAF wild-type and BRAF mutant cells to identify the BRAF pathway inhibitors from microbial metabolites.

First, we observed that ERK1/2 in wild-type BRAF cells was activated by the addition of serum or growth factors, and that ERK1/2 in BRAF-mutated cells is constitutively active (data not shown) by western blotting experiments. Next, we examined the effects of some known inhibitors—actinomycin D (RNA synthesis inhibitor), cycloheximide (protein synthesis inhibitor), paclitaxel (tubulin inhibitor), leptomycin B (CRM1 inhibitor), tunicamycin (*N*-linked glycosylation inhibitor), staurosporine (protein kinase inhibitor), trichostatin A (histone deacetylase inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), LY294002 (PI3 kinase inhibitor), U0126 (MEK inhibitor), geldanamycin (Hsp90 inhibitor), RK-682 (tyrosine phosphatase inhibitor), tautomycin (protein phosphatase 1 and 2A inhibitor) and phoslactomycin C (protein phosphatase 2A inhibitor)—on the cell-based assay.[4,5] We found that U0126 and geldanamycin preferentially decreased the cell viability of BRAF mutant WM266-4 cells (Table 1). The selectivity ratio of CHL-1/WM266-4 was more than 10-fold. The selectivity ratios of other inhibitors were smaller than 1.0. These results suggest that this screening system is suitable for the detection of BRAF-signaling inhibitors from microbial metabolites.

In this cell-based assay, we tested 3000 microbial extracts and identified the aimed inhibitory activity in the fermentation broth of an unidentified fungus. The active compounds were identified as malformin A1, A2 and C[6–9] by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and the amino-acid analysis with Marfey's reagent *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA).[10,11]

Next, we investigated the effects of malformin A1, A2 and C on the growth of BRAF-mutated and wild-type cells in this assay. The IC_{50} values and selectivity ratio of BRAF-nonmutated cells/BRAF-mutated cells of malformin A1, A2 and C are shown in Table 2. Malformin A2 and C preferentially inhibited the growth of BRAF mutant WM266-4 and SK-MEL-28 cells. The selectivity ratio of malformin A1 was lower than that of malformin A2 and C. The difference of amino-acid composition of these three compounds may reflect the difference in biological activity. Structure–activity relationship of malformin derivatives must be studied in the future.

During the investigation of the molecular target, we noticed that malformins changed the cell shapes drastically (Figure 1). WM266-4 shows flat and fibroblastic morphology (Figure 1a), but most of the cells changed to round form after 3 h of treatment with malformin A1, A2 and C (Figures 1c–e).

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It was reported that microcystins and oscillamides, other cyclic peptide compounds, inhibit the protein phosphatases.[12,13] Furthermore, tautomycin, a potent inhibitor of protein phosphatase 1 and 2A, also changed the cell morphology of WM266-4 cells as malformin did (Figure 1b). These results suggest that malformins change cell morphology through protein phosphatase inhibition. To test this possibility, we next investigated the phosphorylation level of vimentin in malformin-treated NIH3T3 cells because it has been known that protein phosphatase 1 and 2A inhibitors induce vimentin overphosphorylation *in situ*.[14,15] To determine whether the phosphorylation sites on vimentin were changed on treatment with malformins, we used site-specific phosphorylated vimentin antibodies, 4A4 and MO82

Table 1 Effect of test compounds on BRAF mutated and wild-type melanoma cell lines

	IC_{50} (μM)		
Compound	WM266-4 (BRAF mutant)	CHL-1 (BRAF wild)	Ratio (CHL-1/WM266-4)
Actinomycin D	0.6	0.04	0.07
Cycloheximide	3.6	0.3	0.08
Paclitaxel	0.2	0.004	0.02
Leptomycin B	0.0016	0.00074	0.46
Tunicamycin	6.0	1.2	0.2
Staurosporine	0.25	0.03	0.12
Trichostatin A	0.05	0.08	1.6
SP600125	50.0	5.5	0.11
SB203580	25.0	40.0	1.6
LY294002	20.0	60.0	3.0
U0126	2.5	30.0	12.0
Geldanamycin	0.04	2.0	50.0
RK-682	67.9	135.9	2.1
Tautomycin	0.07	0.07	1.0
Phoslactomycin C	3.5	4.5	1.3

The concentrations of compounds needed to inhibit the cell growth in 50% of that in WM266-4 and CHL-1 cells were determined as IC_{50}. The selectivity of the inhibition was calculated as the ratio of the IC_{50} value of test compounds treated in CHL-1 cells divided by that in WM266-4 cells.

Table 2 Malformins preferentially inhibited the growth of BRAF-mutated melanoma cell lines

	IC_{50} (μM)			Ratio	
Compound	WM266-4	SK-MEL-28	CHL-1	(CHL-1/WM266-4)	(CHL-1/SK-MEL-28)
Malformin A1	0.05	0.36	0.64	1.2	1.8
Malformin A2	0.83	0.68	5.81	7.0	8.5
Malformin C	0.26	0.08	0.87	3.3	10.9

The concentrations of malformin A1, A2 and C needed to inhibit the cell growth in 50% of that in WM266-4, SK-MEL-28 and CHL-1 cells were determined as IC_{50}. The selectivity of the inhibition was calculated as the ratio of the IC_{50} value of malformin A1-, A2- and C-treated CHL-1 cells divided by that in WM266-4 or SK-MEL-28 cells.

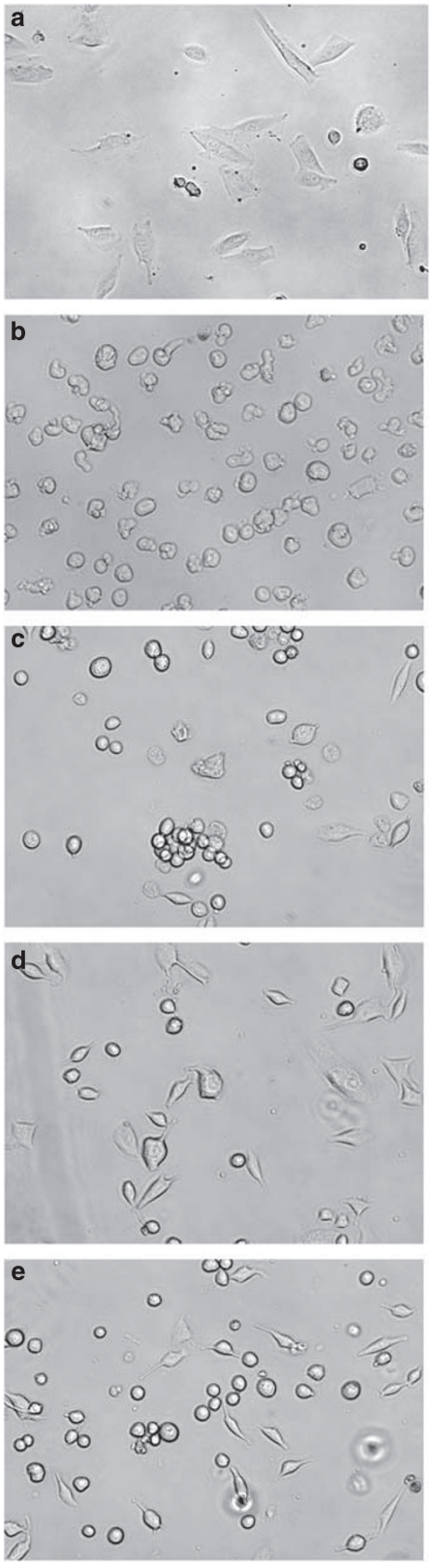

Figure 1 Malformins induced the morphological change in WM266-4 cells. The WM266-4 cells were suspended at a density of 3.0×10^4 cells ml^{-1}. Cell suspension was added to wells of six-well plate (2000 μl per well). Microplates were placed in a CO_2 incubator (air containing 5% CO_2 at 37 °C) for 18 h and morphological change of the cells was observed at 3 h after treatment with the test compounds. (**a**) 1% DMSO, (**b**) 40 μM tautomycin, (**c**) 10 μM malformin A1, (**d**) 20 μM malformin A2 and (**e**) 20 μM malformin C.

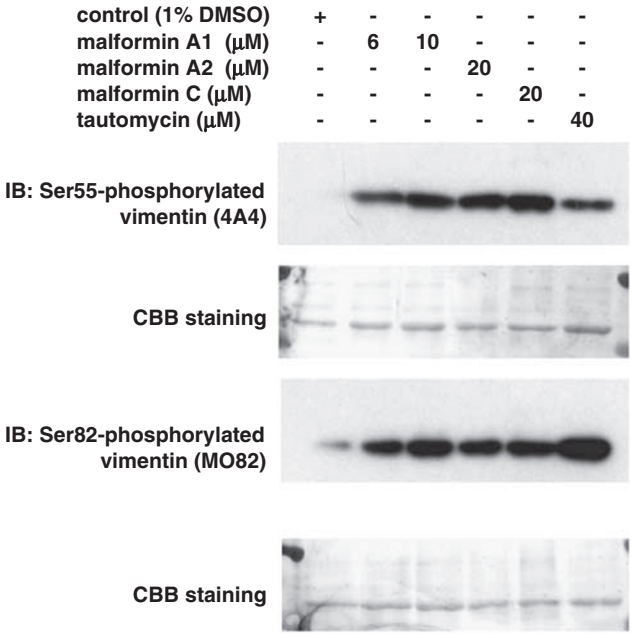

Figure 2 Malformins induced the vimentin phosphorylation of NIH3T3 cells. NIH3T3 cells were treated for 4 h with various concentrations of malformin A1, A2, C and tautomycin. After treatment, the cells were harvested, and immunoblotting was performed. IB: antibodies used for immunoblotting. CBB staining: Coomassie Brilliant Blue solution used for the detection of total vimentin. 1% DMSO treatment (lane 1); 6 μM malformin A1 (lane 2); 10 μM malformin A1 (lane 3); 20 μM malformin A2 (lane 4); 20 μM malformin C (lane 5); 40 μM tautomycin (lane 6).

antibodies that recognize phosphorylation at Ser55 and Ser82, respectively.[15] Tautomycin treatment induced the phosphorylation of Ser55 (4A4) and Ser82 (MO82) of vimentin. Likewise, malformin A1, A2 and C also induced phosphorylation of vimentin at Ser55 and Ser82 in NIH3T3 cells for 4 h incubation (Figure 2). These results suggest that malformin A1, A2 and C may affect protein phosphatases directly or indirectly *in situ*.

There were several reports on the biological activity of malformins,[16–18] but this is the first observation that malformins affected protein phosphorylation of vimentin. Recently, malformin C induced the phosphorylation of cdc2.[19] Our finding is consistent with previous reports.[19–21] Further intensive studies on malformins may shed light on the pivotal events in the BRAF pathway of tumor cells.

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4 Cell culture conditions; WM266-4 (ATCC Number: CRL-1675), SK-MEL-28 (ATCC Number: HTB-72) and CHL-1 cells (ATCC Number: CRL-9446) were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% fetal calf serum and 30 μg/ml of penicillin and 42 μg/ml of streptomycin in 5% CO_2 in an incubator at 37 °C. A Cell-Based Assay conditions; WM266-4, SK-MEL-28 and CHL-1 cells were seeded on 96-well microplates (1.0×10^4 cells per well), respectively. Test compounds were dissolved in methanol at appropriate concentrations and were treated for 72 h at 37 °C in a 5% CO_2 atmosphere. Cell proliferation assays were carried out by the WST-8™ (Nacalai Tesque, Kyoto, Japan) protocol. The absorbance (A450) of each well was measured by a Wallac 1420 multilabel counter (Amersham Biosciences, Piscataway, NJ).
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10 Malformin A1: $[\alpha]^{25}_{D}$ −2.9 (*c* 0.073, MeOH); FAB-MS *m/z* 530 $[M+H]^+$; ESI-MS *m/z* 530 $[M+H]^+$; ESI-MS/MS on $[M+H]^+$ (CE 50 eV) *m/z* 530.4 (100%), 502.5 (22.4%), 417.1 (29.9%), 372.1 (27.1%), 318.1 (11.2 %), 304.1 (23.4%), 258.7 (26.2%), 231.1 (42.1%), 212.9 (18.7%), 199.5 (23.4%), 185.5 (11.2%); HR FAB-MS calcd. for $C_{23}H_{40}N_5O_5S_2$ $[M+H]^+$ 530.2471, found: 530.2528; molecular formula: $C_{23}H_{39}N_5O_5S_2$; Amino acid anlysis (ratio): L-Val(1), D-Leu(1), L-Ile(1). Malformin A2: $[\alpha]^{25}_{D}$ +1.7 (*c* 0.039, MeOH); FAB-MS *m/z* 516 $[M+H]^+$; ESI-MS *m/z* 516 $[M+H]^+$; ESI-MS/MS on $[M+H]^+$ (CE 50 eV) *m/z* 516.4 (100%), 488.2 (26%), 471.4 (29%), 417.0 (41%), 403.0 (14%), 372.2 (32%), 304.2 (45%), 259.0 (25.0%), 231.0 (47.0%), 213.0 (25.0%), 185.0 (28.0%); HR FAB-MS calcd. for $C_{22}H_{38}N_5O_5S_2$ $[M+H]^+$ 516.2314, found: 516.2309; molecular formula: $C_{22}H_{37}N_5O_5S_2$; Amino acid analysis (ratio): L-Val(2), D-Leu(1). Malformin C: $[\alpha]^{25}_{D}$ −1.5 (*c* 0.20, MeOH); FAB-MS *m/z* 530 $[M+H]^+$; ESI-MS *m/z* 530 $[M+H]^+$; ESI-MS/MS on $[M+H]^+$ (CE 50 eV) *m/z* 530.4 (100%), 502.2 (15.9%), 485.4 (19.0%), 417.2 (31.7%), 372.2 (28.6%), 318.2 (15.9%), 304.2 (30.2%), 259.2 (21.4%), 230.8 (36.5%), 213.4 (12.7%), 199.2 (11.9%), 185.2 (18.3%); HR FAB-MS calcd. for $C_{23}H_{40}N_5O_5S_2$ $[M+H]^+$ 530.2471, found: 530.2536; molecular formula: $C_{23}H_{39}N_5O_5S_2$; Amino acid analysis (ratio): L-Val(1), L-Leu(1), D-Leu(1).
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