Isolation from *Streptomyces* of a Novel Naphthoquinone Compound, Naphthablin, That Inhibits Abl Oncogene Functions*

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In the course of our screening for inhibitors of abl oncogene function, a culture filtrate of *Streptomyces aculeolatus*. induced normal flat morphology in v-abl-expressing NIH3T3 cells. The active substance was isolated through ethyl acetate extraction, silica gel column chromatography, and reverse-phase HPLC. Mass and NMR spectroscopy including HMBC revealed that it had a novel naphthoquinone structure with a monoterpene, and we named it "naphthablin". Naphthablin inhibited Abl-induced morphological transformation in v-abl^{ts}-NIH3T3 cells at around 30 μg/ml, and specifically inhibited RNA synthesis.

In human chronic myelogenous leukemia (CML), the cellular oncogene abl is activated by chromosomal translocation to give bcr-abl[1]. The product of bcr-abl or viral abl has higher tyrosine kinase activity than that of unactivated cellular abl[2]. A tyrosine kinase inhibitor, herbimycin, was shown to induce erythroid differentiation in human CML K562 cells, and also showed antitumor activity against abl-expressing tumors in mice[3]. Recently, we isolated aristeromycin from *Actinomycetes* as an inhibitor of v-abl functions[4]. Aristeromycin induced normal morphology in v-abl^{ts}-NIH3T3 cells and erythroid differentiation in K562 cells. It did not inhibit cellular tyrosine phosphorylation, but inhibited methylation reactions such as the conversion of phosphatidylethanolamine to phosphatidylcholine in K562 cells.

As anti-Abl compounds may become effective chemotherapeutic agents for use against CML, we have been searching among microbial metabolites for inhibitors of abl function. In the course of our screening for such inhibitors, we have isolated a novel naphthoquinone compound from *Streptomyces aculeolatus*, which inhibitor is described herein.

Materials and Methods

Cell Culture

v-abl^{ts}-NIH3T3 cells[5] were kindly supplied by Dr. J. WANG, UCSD, San Diego. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at the permissive temperature (33°C) or the nonpermissive temperature (39°C) in a 5% CO_2-95% air atmosphere.

Fermentation of *Streptomyces* sp. MK15-42F22

Strain MK15-42F22 was isolated from a soil sample collected in Sakata, Yamagata Prefecture, Japan, in 1993. The strain has been deposited in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan, under the accession number FERM P-14635. Spores of strain MK15-42F22 were inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a medium composed of 2.0% soluble starch, 2.5% wheat germ, 0.3% calcium carbonate, 0.15% sodium chloride, and 0.01% wheat germ oil (pH 7.2) and cultured at 27°C for 5 days on a rotary shaker at 180 rpm. Two milliliters of the seed culture was inoculated in each of ten 500-ml Erlenmeyer flasks containing 100 ml of a medium composed of 2.4% soluble starch, 0.3% meat extract, 0.5% tryptose, 0.5% yeast extract, 0.1% glucose and 0.2% calcium carbonate (pH 7.4); and the microorganisms were cultured at 27°C for 6 days.

Isolation of Naphthablin

The cultured broth (1 liter) was centrifuged at 10,000 rpm for 10 minutes. Then the supernatant was extracted with an equal amount of ethyl acetate, and the organic layer was evaporated. The precipitate residue of

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the broth was extracted with 300 ml of acetone, and the acetone extract was added by water. After removal of the acetone, the solution was extracted with ethyl acetate, and the extract was concentrated to dryness. The supernatant fraction was combined with the precipitate fraction, then the combined material was dissolved in a small volume of $CHCl_3$, and applied to a silica gel column. Then, the active fraction was eluted with hexane-ethyl acetate (4:1~3:1). The crude material was dissolved in MeOH and further purified by reverse-phase HPLC (Senshu Pak, Pegasil OUJ-1251-N4, 6 i.d. × 250 mm) with 90% MeOH containing 0.1% trifluoroacetic acid to give 11 mg of a pure orange material.

Results

In the course of our screening for abl oncogene function inhibitors, we found that the culture filtrate of strain MK15-42F22 induced normal flat morphology in v-abl^{ts}-NIH3T3 cells at the permissive temperature. The morphology of the producing strain is shown in Fig. 1. Taxonomic studies indicated that the producing strain belongs to *Streptomyces aculeolatus*[6].

Production of the anti-Abl compounds remained at the same level from 5 to 10 days of cultivation. Therefore, we took the broth on day 5. The active principle was extracted with ethyl acetate, and purified as described in Materials and Methods.

The structure of the active compound was elucidated by analysis of mainly mass and two-dimensional NMR spectra. Since the structure is novel, as shown in Fig. 2,

Fig. 1. Scanning electromicrograph of the producing strain, *Streptomyces aculeolatus*. MK15-42F22.

Bar represents 1 μm.

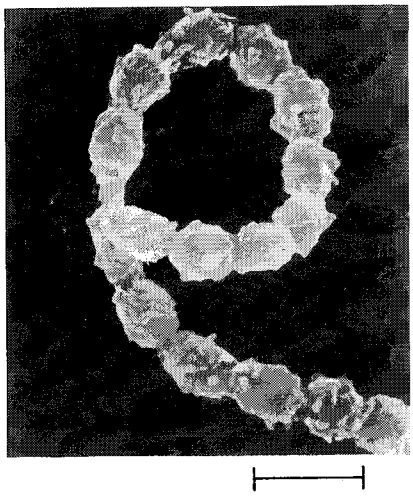


Fig. 2. Structure of naphthablin.

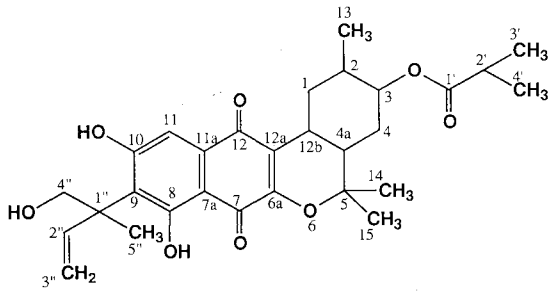


Table 1. Physico-chemical properties of naphthablin.

Appearance	Orange powder
Molecular formula	$C_{29}H_{36}O_8$
FAB-MS (*m/z*)	positive: 513 $(M+H)^+$,
	negative: 511 $(M-H)^-$
HRFAB-MS (*m/z*)	Found: 513.2490 $(M+H)^+$
	Calcd: 513.2488 (for $C_{29}H_{37}O_8$)
$[\alpha]_D^{24}$	-166° (*c* 0.2, $CHCl_3$)
IR υ_{max}^{KBr} cm^{-1}	3410, 2980, 2944, 2895,
	1736, 1655, 1638, 1580
UV λ_{max}^{MeOH} nm (ε)	219.6 (21960), 271.6 (15656),
	315.2 (10631), 421.6 (4010)
UV $\lambda_{max}^{MeOH\text{-}0.1N\ NaOH}$ nm (ε)	213.2 (45310), 232.8 (23868),
	298.4 (18530), 330.4 (8157),
	432.4 (3584), 514.4 (4442)
Rf value[a]	
$CHCl_3$-MeOH (20:1)	0.67
toluene-acetone (3:1)	0.48
hexane-ethyl acetate (1:1)	0.68

a) silica gel TLC (Merck $60F_{254}$)

Table 2. Chemical shifts of naphthablin (carbon-13, $CDCl_3$).

No.	δ_C (125 MHz*)		
1	28.7	13	17.6
2	30.4	14	25.1
3	71.8	15	25.8
4	28.0	1'	176.8
4a	36.4	2'	34.5
5	80.8	3'	19.1
6a	155.8	4'	19.2
7	182.4	1"	47.1
7a	108.2	2"	143.7
8	164.0	3"	115.8
9	121.3	4"	68.8
10	163.4	5"	22.0
11	112.5		
11a	132.7		
12	183.5		
12a	121.3		
12b	30.3		

* Chemical shifts in ppm from TMS as an internal standard.

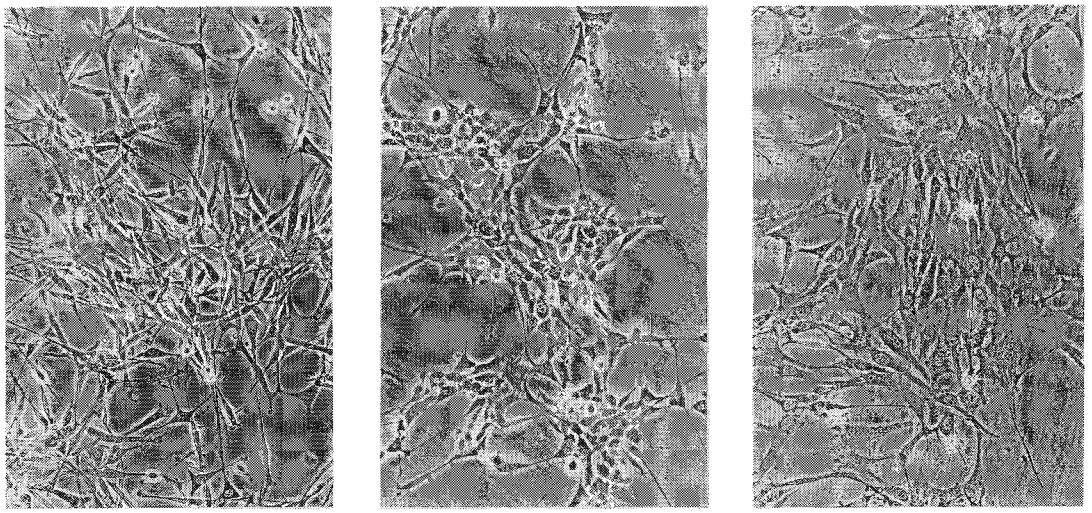
Table 3. Chemical shifts of naphthablin (proton, $CDCl_3$).

No.	δ_H (500 MHz*)		
1	1.73 (1H, ddd, J=13.6, 13.6, 5.0 Hz)	13	0.82 (3H, d, J=6.8 Hz)
	2.82 (1H, ddd, J=13.6, 3.2, 3.2 Hz)	14	1.31 (3H, s)
2	1.44 (1H, m)	15	1.46 (3H, s)
3	4.92 (1H, m)	2'	2.60 (1H, qq, J=7.0, 7.0 Hz)
4	1.32 (1H, m)	3'	1.20 (3H, d, J=7.0 Hz)
	2.02 (1H, ddd, J=14.6, 3.4, 3.4 Hz)	4'	1.21 (3H, d, J=7.0 Hz)
4a	1.87 (1H, ddd, J=12.8, 5.0, 5.0 Hz)	2"	6.13 (1H, dd, J=17.6, 10.6 Hz)
11	7.12 (1H, s)	3"	5.12 (1H, d, J=17.6 Hz)
12b	3.22 (1H, ddd, J=3.2, 5.0, 5.0 Hz)		5.33 (1H, d, J=10.6 Hz)
		4"	3.69 (1H, d, J=13.8 Hz)
			4.08 (1H, d, J=13.8 Hz)
		5"	1.52 (3H, s)
		8-OH	13.35 (1H, s)

* Chemical shifts in ppm from TMS as an internal standard.

Fig. 3. Inhibition of Abl-induced morphological transformation by naphthablin in v-abl^{ts}-NIH3T3 cells.

The cells were incubated at 39°C for 24 hours, then, they were incubated at 33°C for 24 hours without (A) or with (B) 0.3 μg/ml of naphthablin. The cells at 33°C without the inhibitor were further incubated for 24 hours at 39°C without any additive (C).



A B C

Table 4. Growth inhibition of cultured cell lines by naphthablin.

Cell line		Growth inhibition* (IC_{50}, μg/ml)
v-abl^{ts}-NIH3T3	(33°)	7.6
	(39°)	17.5
RSV-NIH3T3		11.2
NIH3T3		12.6
K562		1.5

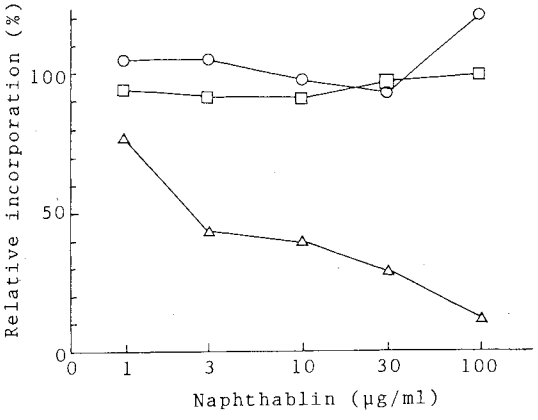
* Adherent cells (2×10^4/48-well plate) were incubated with naphthablin for 3 days in DMEM containing 5% calf serum. K562 cells (3×10^4/24-well plate) were incubated for 4 days in RPMI 1640 medium supplemented with 10% fetal bovine serum.

we named the compound "naphthablin". Physicochemical properties of naphthablin are listed in Table 1, and the ^{13}C and ^{1}H NMR data appear in Tables 2 and 3, respectively. The core structure of naphthablin, having naphthoquinone and a monoterpene, is related to those structures of naphterpin[7] and naphthgeranine A[8]. Among them, naphthablin is the most complicated, and it has a substituted propenyl group and an isobutyrate at the 9 and 3 positions, respectively.

Naphthablin inhibited Abl-induced morphological transformation in v-abl^{ts}-NIH3T3 cells at concentrations of 20~40 μg/ml. Morphological transformation could be induced in 24 hours by the temperature shift from

Fig. 4. Effect of naphthablin on macromolecular synthesis in v-abl^{ts}-NIH3T3 cells.

The cells were incubated with naphthablin at 33°C in the presence of labeled thymidine (○), uridine (△) or leucine (□) for 1 hour.



39°C to 33°C in v-abl^{ts}-NIH3T3 cells, as shown in Figs. 3A and 3C. Addition of 30 μg/ml of naphthablin inhibited this transformation, and maintained the flat morphology (Fig. 3B). The effect of naphthablin on the growth of cultured cells is shown in Table 3. The IC_{50} values were around 10 μg/ml in most cell lines. Human chronic myelogenous leukaemia K562 cells were especially sensitive to naphthablin. The effect of naphthablin on DNA, RNA, and protein synthesis is shown in Fig. 4. Naphthablin inhibited only RNA synthesis at 3～100 μg/ml.

Discussion

Naphthablin has a unique naphthoquinone structure, although its stereochemistry is not known. Naphterpin, having the same core structure, was isolated from *Streptomyces* as an antioxidative agent[9]. Whether or not naphthablin has an antioxidative effect is not known yet.

Naphthablin induced flat morphology in v-abl^{ts}-NIH3T3 and RSV-NIH3T3 cells but not in K-ras-NIH3T3 cells. The mechanism of the anti-Abl effect is not understood yet. But when the v-abl^{ts}-NIH3T3 cells were incubated with 30～50 μg/ml of naphthablin, the intracellular tyrosine phosphorylation was dose-dependently lowered. The amount of Abl also decreased at these same concentrations. Therefore, naphthablin may induce normal phenotypes possibly by inhibiting Abl synthesis or decreasing the stability of Abl.

Unlike aristeromycin[4], naphthablin did not induce erythroid differentiation in human CML K562 cells, possibly because naphthablin shows cytotoxicity toward K562 cells at lower concentrations, as shown in Table 3.

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