BIOLOGICAL ACTIVITIES OF IC201 ((3*S*,8*E*)-1,3-DIHYDROXY-8-DECEN-5-ONE), A LOW MOLECULAR WEIGHT IMMUNOMODULATOR PRODUCED BY *STREPTOMYCES*

SHIGETOSHI MIZUTANI, HIDEHARU ODAI, TORU MASUDA, MASATOMI IIJIMA, MICHIYO OSONO, MASA HAMADA†, HIROSHI NAGANAWA†, MASAAKI ISHIZUKA* and TOMIO TAKEUCHI†

Institute for Chemotherapy,
18-24 Motono, Miyamoto, Numazu, Shizuoka 410-03, Japan
†Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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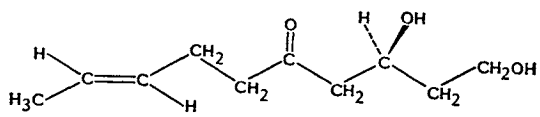
IC201 was found in cultured broth of *Streptomyces cirratus* as an antitumor antibiotic which was effective in retarding growth of the established solid tumor of Ehrlich carcinoma by treatment starting 8 days after tumor inoculation. It retarded growth of the established solid tumor of IMC carcinoma but had no cytotoxicity at 100 μg/ml. IC201 treatment kept NK cell activity of tumor-bearing mice at normal level and stimulated cytostatic activity of peritoneal macrophages. It stimulated phagocytosis of yeast and phorbol myristate acetate-elicited superoxide production by peritoneal macrophages. The addition of IC201 to P388D_1 cell cultures enhanced release of interleukin 1 (IL-1) into cultured supernatant but it affected lipopolysaccharide-induced IL-1 production. Although the addition to macrophage-depleted cultures did not show any stimulatory effect, mixed lymphocyte culture reaction was augmented in cultures using spleen cells as stimulator cells taken from mice given IC201. Results indicate that IC201 primarily activates macrophages and the activation may cause modulation of immune responses.

In the course of screening for antitumor substances from microbial sources which have activity against an established solid tumor, we found a low molecular weight substance, IC201, in cultured broth of *Streptomyces* sp. MH663-mF3. The structure was determined to be (3*S*,8*E*)-1,3-dihydroxy-8-decen-5-one (Fig. 1) on the basis of spectroscopic evidence. IC201 was identical to the substance reported by KELLER-SCHIERLEIN *et al.*[1] who isolated it from cultured broth of *Streptomyces fimbriatus* as a positive substance to Ehrlich-reagent. They did not report biological activities except for inactivity against bacteria, yeasts and fungi. We found that IC201 showed antitumor effect against a solid tumor through activation of host mediated events. In this paper we report biological activities of IC201.

Fig. 1. Structure of IC201.



Materials and Methods

The Producing Organism and Fermentation

Strain MH663-mF3 was isolated from a soil sample collected in the premises of the Institute of Microbial Chemistry, Shinagawa-ku, Tokyo and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession No. FERM P-9710. Taxonomy of the producing organism was

performed by the method of SHIRLING and GOTTLIEB[2]. Diaminopimelic acid was determined according to the method reported by BECKER *et al.*[3].

Morphological, cultural and physiological characteristics of strain MH663-mF3 indicated that the strain belonged to the genus *Streptomyces* and was especially close to *Streptomyces humidus*, *Streptomyces griseolavendus* and *Streptomyces cirratus*[3]. The color of hygroscopic aerial mycelium and the ability of *S. humidus* to produce melanoid pigment were different from MH663-mF3. *S. griseolavendus* was also different from MH663-mF3 in the utilization of L-arabinose, D-xylose, sucrose, inositol, rhamnose and D-mannitol. Therefore, the properties of *S. cirratus* IMC S-0751 (ISP 5479) were compared with that of the strain MH663-mF3. Morphological, cultural and physiological characteristics of *S. cirratus* were very close to the strain MH663-mF3, although the inositol and D-mannitol utilization was slightly different and sucrose utilization was not clear in the literature[4]. From these results, the strain MH663-mF3 was identified with *S. cirratus*.

Fermentation was done as follows. The seed medium contained galactose 2.0%, dextrin 2.0%, Bacto-soytone 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, silicone one drop, pH adjusted to 7.4; the production medium contained glycerol 3.0%, fish powder 2.0%, $CaCO_3$ 0.2%, pH adjusted to 7.4.

Growth from a well-grown agar slant of the strain MH663-mF3 was inoculated into 110 ml of the seed medium in 500-ml Erlenmeyer flasks and cultured at 30°C for 48 hours on a rotary shaker (180 rpm). Two ml of the above seed culture was inoculated into 130 ml of the production medium in 500-ml Sakaguchi flasks and incubated at 27°C for 120 hours on a reciprocal shaker (130 times per minute).

Isolation and Determination of the Structure

The cultured broth (30 liters) was filtered and activated carbon (270 g) was added. After filtration and washing with water, the active crude material against Ehrlich carcinoma was extracted with MeOH (4 liters), evaporated *in vacuo*, dissolved in water (pH 8.0), and extracted with butanol. The solvent layer was evaporated and the crude material was applied to a column of Wakogel C-200 (20×160 mm). After washing the column with a mixture of hexane and ethyl acetate (5:5), the active fraction was eluted with a mixture of hexane and ethyl acetate (35:65) and concentrated *in vacuo* to give brown oily material (110 mg). The resulting residue was applied to a reverse phase HPLC column (Senshu pak. ODS 20×300 mm, flow rate 5 ml/minute) and eluted with a linear gradient of 25 to 30% CH_3CN (70 minutes). The active fraction was concentrated *in vacuo* and applied to a column of Wakogel C-200 (20×160 mm). This was followed by elution with a mixture of hexane and ethyl acetate (35:65) and the active fraction was concentrated to dryness giving IC201 (49.1 mg) as colorless oil.

The structure of IC201 was determined to be 1,3-dihydroxy-8-decen-5-one on the basis of spectroscopic evidence (^{1}H NMR, ^{1}H-^{1}H correlation spectroscopy (COSY) 2D NMR, ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT) and ^{1}H-^{13}C COSY 2D NMR spectra). Configuration for 3-position was determined as *S* on the basis of the specific rotation[1]. IC201 has (*E*)-configuration which was indicated by ^{1}H NMR coupling constant ($J_{8,9}$=15 Hz). This coupling constant was calculated from the ^{1}H NMR spectrum of acetylated IC201 in C_6D_6 which was double irradiated at 1.53 ppm (10-H) and 2.16 ppm (7-H).

IC201 was dissolved at high concentration (2 or 4 mg/ml) in 10% DMSO solution and diluted with saline or medium for experiments. The vehicle containing DMSO (less than 0.1% in final) was added or injected to the control in each experiment.

Animals

C3H/He, C57BL/6, BALB/c, CDF_1 and ICR female mice, 6 to 8 weeks old, were purchased from Charles River Japan Inc., Japan. They were maintained under specific pathogen-free conditions at 23±1°C and 55±5% humidity.

Antitumor Activity

For assay of IC201 activity during purification and isolation, Ehrlich carcinoma (solid tumor)

was employed. ICR mice (female, 6 weeks old) were inoculated sc with 1×10^6 Ehrlich carcinoma cells. Seven days later mice were randomized and given IC201 days 8 to 13. The mean tumor weight was determined day 14 after the inoculation.

IMC carcinoma was maintained in CDF_1 mice by weekly ip injection. To determine the antitumor effect on IMC carcinoma bearing mice, CDF_1 mice were inoculated sc with 1×10^6 tumor cells and IC201 was administered ip every other day or daily in different schedules. Mean tumor weight was determined 28 days after the inoculation.

The mean of percentage of inhibition was calculated as follows:

$$\text{Inhibition (\%)}=\left(1-\frac{\text{Mean tumor weight of treated group}}{\text{Mean tumor weight of control group}}\right)\times100$$

NK Activity

NK activity of spleen cells of CDF_1 mice treated with IC201 was determined at 30 days after transplantation of IMC carcinoma. YAC-1 cells, used as a target cell, were labeled with ^{51}Cr, and 2×10^5 cells/ml were incubated with effector cells at ratios of 75 : 1 for 4 hours. After incubation the supernatant was collected and ^{51}Cr radioactivity was counted in a gamma counter (Aloka Co., Ltd., Tokyo). The maximum counts in target cells were determined after disruption by freezing and thawing. The mean percentage of specific cytotoxicity was calculated as follows:

$$\text{Cytotoxicity (\%)}=\frac{\text{Test count}-\text{Spontaneous count}}{\text{Maximum count}-\text{Spontaneous count}}\times100$$

Macrophage Functions

To determine the cytostatic activity of peritoneal exudate cells of IC201 treated mice, IC201 (5 mg/kg, ip) was injected 7, 5 or 3 days before harvest of cells. CDF_1 mice were injected ip with 1.5 ml of 10% Proteose peptone, 3 days thereafter, peritoneal exudate cells were taken and adherent cells were prepared. Adherent cells (2×10^5 cells/culture) were cultured with EL-4 cells (1×10^4 cells/culture) for 24 hours and the incorporation of [^{3}H]thymidine ([^{3}H]TdR) into EL-4 cells for 6 hours was determined.

The influence of IC201 on the release of phorbol myristate acetate (PMA)-elicited superoxide anion from peritoneal macrophages was examined using the method reported by KITAGAWA and JOHNSTON[5]. Briefly, mice were injected with IC201, 1 day before assay, and peritoneal exudate cells were collected. The peritoneal exudate cells were incubated in a plastic dish for 2 hours at 37°C in 5% CO_2 in air and adherent cells were prepared. Then, adherent cells were incubated with PMA for 1 hour and superoxide anion content in the supernatant was determined by measuring reduction of ferricytochrome C.

Effect of IC201 on phagocytosis of thioglycolate-stimulated peritoneal macrophages was examined as reported before[6]. Briefly, mice were given IC201 3 days or 1 day before assay, thioglycolate-stimulated peritoneal exudate cells were collected and adherent cells were prepared as mentioned above. To adherent cells, 0.05 ml of yeast suspension (7.5×10^6 cells/ml) was added and incubated for 45 minutes at 37°C in 5% CO_2 in air. After washing thoroughly, adherent cells on the bottom of the plates were stained with May-Grunwald and Giemsa solution and the number of phagocytic cells were counted. Triplicate cultures were done for each data point.

Production of Interleukin 1 (IL-1) by $P388D_1$ Cell Line

$P388D_1$ cells (1×10^6 cells/ml) were incubated with or without 10 μg/ml of lipopolysaccharide (LPS) at 37°C for 4 hours and then washed with medium three times. After washing, cells were resuspended in medium at 4×10^5 cells/ml and cultured with various concentrations of IC201 for 24 hours. The culture supernatant was obtained and dialyzed against RPMI 1640 without fetal calf serum (FCS) and 2-mercaptoethanol (2ME) for 20 hours, and the non-dialyzable fraction was used for the determination of IL-1 activity. Activity of IL-1 was assayed according to the method described by MIZEL[7]. Briefly, thymocytes were taken from C3H/He female mice (6~8 weeks old) and suspended at 1.5×10^7 cells/ml in RPMI 1640 containing 10% FCS, and 2.5×10^{-5} M of 2ME. The dialyzed supernatants (100 μl) from cultured $P388D_1$ cells and 100 μl of cell suspension in flat-

bottom, 96-well microplates were cultured with 1 μg/ml phytohemagglutinin-P (PHA-P) at 37°C for 72 hours in 5% CO_2 in air. The cultures were pulsed with [^{3}H]TdR 16 hours before assay, and the radioactivity of cultured cells was counted by a scintillation counter. Triplicate determinations were made.

Mixed Lymphocyte Culture Reaction (MLCR)

MLCR was tested by a commonly used method[8]. Spleen cells taken from C3H/He mice (H-2^k) used as a responder (cells passed through nylon wool column) were mixed with stimulator spleen cells taken from BALB/c (H-2^d) (given IC201 as indicated in tables). The stimulator cells were irradiated with 470 R/minute for 10 minutes[9]. Nylon wool passed-spleen cells of BALB/c mice were used as macrophagedepleted stimulator cells. The mixed cells were cultured in medium containing 5% FCS at 37°C for 5 days in 5% CO_2 in air and [^{3}H]TdR was added to each well 16 hours before cell harvest. MLCR was determined by measuring the incorporation of [^{3}H]TdR into the cultured cells. Triplicate determinations were made.

Production of Interleukin 2 (IL-2) in MLC

To measure IL-2 activity in supernatants of MLC, the supernatant was collected 72 hours after the start of MLC and dialyzed against RPMI 1640 for 24 hours, and the non-dialyzable fraction was used. IL-2 activity was measured by the method previously described[8]. Briefly, nylon wool-passed lymphocytes were prepared from spleen cells of CDF_1 mice and cells (1×10^7 cells/ml) were preincubated with concanavalin A (Con A, 5 μg/ml) and washed thoroughly after addition of α-methyl-D-mannopyranoside (20 mg/ml). The Con A-pretreated blastoid cells were resuspended (2×10^6 cells/ml) in RPMI 1640 containing 5% FCS and 2.5×10^{-5} M of 2ME. The cell suspension (100 μl) and supernatants (100 μl) from MLC was added into wells of a microplate. After 72 hours incubation at 37°C in 5% CO_2, the incorporation of [^{3}H]TdR into blastoid cells was counted by a liquid scintillation counter ([^{3}H]TdR pulsed 16 hours before assay). Triplicate determinations were made.

Statistical Analysis

Results were analyzed for statistical significance by Student's t-test.

Results

Antitumor Activity

IC201 exhibited antitumor activity against the established solid tumor of IMC carcinoma over a wide dose range. CDF_1 mice were implanted with 1×10^6 IMC carcinoma cells sc and IC201 was given ip every other day starting day-1 or day-8 after implantation. As shown in Table 1, IC201 re-

Table 1. Antitumor activity of IC201 against IMC carcinoma.

IC201 (mg/kg/day, ip)	Schedule (days)			
	1~27		8~27	
	Mean tumor weight (mg±SD)	Inhibition (%)	Mean tumor weight (mg±SD)	Inhibition (%)
100	1,915.8±355	42.7***	1,775.5±897	46.9**
50	2,238.5±500	33.1***	1,732.6±792	48.2***
25	1,545.8±575	53.8***	1,992.2±767	40.4***
12.5	2,459.2±614	26.5*	1,793.8±146	46.7***
6.3	2,568.4±757	23.2*	2,716.6±726	18.8
3.1	1,837.0±646	45.1***	2,944.0±453	12.0
0	3,345.0±469	—	3,345.0±469	—

CDF_1 mice were inoculated with IMC carcinoma cells (1×10^6 cells/mouse, sc). Mean tumor weight was determined 28 days after inoculation. IC201 was administered ip every other day on the indicated days.

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table 2. NK activity of spleen cells in tumor-bearing mice after IC201 treatment.

Mice	Mean tumor wieght (mg±SD)	Inhibition (%)	Spleen weight (mg±SD)	NK activity (%)
Normal	—	—	87±5	7.6±2.2
Tumor-bearing	8,130.5±285.0	—	574±69	1.4±0.5
Tumor-bearing IC201 treated	2,859.0±87.7	64.8***	346±71	6.3±2.4*

CDF_1 mice were implanted with IMC carcinoma (1×10^6 cells/mouse, sc). IC201 (50 mg/kg/day) was administered ip day-7, 8, 9, 10, 11 and 12 after inoculation. Mean tumor weight was determined 30 days after implantation. NK activity was determined against ^{51}Cr-labeled YAC-1 cells at a ratio of 75 : 1.
* $P<0.05$, *** $P<0.001$.

tarded the growth of the solid tumor by 33.1~53.8% at 25 to 100 mg/kg/day on both schedules. Further, IC201 retarded the growth of Ehrlich carcinoma (solid type) although it did not increase the survival period of mice implanted with Ehrlich ascites tumor and L1210 leukemia cells (data not shown). At 100 μg/ml, IC201 had no antimicrobial and cytotoxic activity against cultured L1210 leukemia cells and IMC carcinoma cells. Single intravenous injection of 500 mg/kg of IC201 into ICR mice did not show toxicity. There results suggested that the antitumor activity of IC201 may have been due to activation of host mediated mechanisms without direct cytotoxicity to tumor cells.

Table 3. Cytostatic activity of peritoneal macrophages taken from mice given IC201 against EL-4 lymphoma cells.

Schedule (days)	cpm/culture±SD	Inhibition (%)
Control	76,170±7,978	
−7	57,747±3,367	24.2**
−5	59,234±5,763	22.2*
−1	45,521±1,595	40.2**

CDF_1 mice were injected with 1.5 ml of 10% Proteose peptone, 3 days thereafter, peritoneal exudate cells were taken and adherent cells (2×10^5 cells/culture) were cultured with EL-4 cells (1×10^4 cells/culture) for 24 hours. IC201 (5 mg/kg, ip) was injected 7, 5 or 3 days before harvest of cells. The incorporation of [^{3}H]TdR into EL-4 cells was determined.
* $P<0.05$, ** $P<0.01$.

We examined NK activity of spleen cells taken from tumor-bearing mice given IC201. IC201 was administered ip to CDF_1 mice implanted with IMC carcinoma cells, daily from day-7 to day-12 after implantation and NK activity was determined against ^{51}Cr-labeled YAC-1 cells at a ratio of 75 : 1. As shown in Table 2, although NK activity in non-treated tumor-bearing mice was reduced markedly, IC201 treatment inhibited tumor growth and kept NK activity at normal level. However, IC201 had

Table 4. Production of superoxide anion and phagocytosis of yeasts by peritoneal macrophages of mice given IC201.

IC201 (mg/kg, ip)	PMA-induced O^{2-} (nmol/mg protein±SD)[a]	T/C (%)	Phagocytosis No. of phagocytic cells (±SD)[b]	T/C (%)
25	78.6±7.8**	165	121.6±10.1***	200
6.2	96.6±9.3**	203	91.5±7.2**	151
1.6	81.7±8.0**	171	96.4±9.7**	159
0.4	78.5±1.5*	165	93.7±8.0**	154
0	47.7±10.7	100	60.8±5.1	100

[a] CDF_1 mice were given IC201 ip, 1 day before harvest of cells. Activity of superoxide anion determined by the method of reduction of ferricytochrome C.
[b] Mice were given IC201 ip, 1 day before harvest of cells. The number of phagocytic cells was counted in 400 macrophages.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

no effect on NK activity of normal mice (data not shown).

We examined the effect of IC201 on cytostatic activity of peritoneal macrophages. Peritoneal macrophages taken from mice given IC201 (5 mg/kg, ip) 7, 5 or 3 days before assay were cultured and their cytostatic activity against growth of EL-4 cells was determined. As shown in Table 3, peritoneal macrophages taken from mice given IC201 inhibited the incorporation of [^{3}H]TdR into EL-4 cells at 22.2~40.2%. This indicates that IC201 stimulated cytostatic activity of peritoneal macrophages in mice. Moreover, we obtained the result that the antitumor activity of IC201 was reduced markedly in carrageenan-treated mice.

Macrophage Function

The effect of IC201 on macrophage functions was investigated. Release of superoxide and stimulation of phagocytosis are known as parameters of macrophage activation. Thus, the effect of IC201 on them was examined. CDF_1 mice were given IC201 ip once, 1 day before sacrifice. As shown in Table 4, the production of superoxide anion was enhanced and the number of phagocytic cells was increased by administration of IC201 at doses greater than 0.4 mg/kg. In these experiments, the number of peritoneal exudate cells and plastic dish-adherent macrophages were not changed significantly by the administration of IC201 (data not shown). Results suggest that IC201 may differentiate normal macrophages into primed macrophages[10].

Cytokine Production

Effect of IC201 on release of IL-1 from $P388D_1$ cells was examined. As shown in Table 5, release of IL-1 from $P388D_1$ cells was enhanced at 1 or 10 μg/ml of IC201, but not at 100 μg/ml. On the other hand, no augmentation of the release from LPS-pretreated $P388D_1$ cells was observed. In this case, at a dose of 100 μg/ml release was suppressed.

Table 5. Effect of IC201 on release of IL-1 from $P388D_1$ cells.

Cells treated with IC201	IC201 (μg/ml)	[^{3}H]TdR incorporation	
		cpm±SD	T/C (%)
$P388D_1$	100	470±111	127
	10	989±169**	267
	1	1,629±321**	440
	0	370±69	100
LPS-pretreated $P388D_1$	100	3,640±357*	71.2
	10	4,401±629	86.1
	1	6,231±740	122
	0	5,112±933	100

Release of IL-1 was done by incubation of $P388D_1$ cells or LPS-pretreated $P388D_1$ cells with IC201. IL-1 was assayed using thymocytes as the target cells in the presence of PHA.

* $P<0.05$, ** $P<0.01$.

Table 6. MLCR with non-treated or macrophage (Mϕ)-depleted spleen cells as a stimulator taken from mice given IC201.

mg/kg/day	cpm±SD		
	Non-treated		Mϕ-depleted MLCR
	MLCR	IL-2 activity	
0	3,240±105	3,978±12	6,106±810
1.3	3,854±1,568	4,343±521	4,346±179
5	10,370±1,461*	13,527±378*	4,247±194
20	10,030±1,468*	3,212±145	6,049±995

Spleen cells taken from mice given IC201, 4, 3 and 2 days before harvest were used as a stimulator cells. Non-treated or nylon wool passed (Mϕ-depleted) spleen cells were irradiated with 470 R/minute for 10 minutes.

* $P<0.05$, ** $P<0.01$.

Next, we examined the effect of IC201 on MLCR. Although the addition of IC201 to MLC did not show any stimulatory effect, we tested the effect of IC201 on MLCR using spleen cells, which contained macrophages as stimulator cells, taken from mice given IC201. The stimulator cells were irradiated with 470 R/minute for 10 minutes[9]. Results are shown in Table 6. MLCR was enhanced in cultures of the stimulator cells taken from mice given 5 or 20 mg/kg/day of IC201 but not in cultures with macrophage-depleted stimulator cells. IL-2 activity in cultures with nontreated stimulator cells was examined. As shown in Table 6, IL-2 production was augmented only at 5 mg/kg/day, and it was same as normal level at 20 mg/kg/day.

Discussion

IC201 was found to be an antitumor antibiotic against an established solid tumor. In screening, the antitumor activity of IC201 was determined by treatment starting on day-8 after tumor inoculation, daily for 4 or 7 days. As shown in Table 1, IC201 exhibited moderate antitumor effect against IMC carcinoma, a syngeneic tumor, on both schedules starting 1 day or 8 days after tumor inoculation. IC201 showed slight cytotoxicity to tumor cells at only high concentration, more than 100 μg/ml. As we examined the influence of IC201 on lymphocyte functions such as lectin-induced blastogenesis and T cell proliferation, it did not show any effects except a weak inhibition at more than 25 μg/ml. Therefore we thought that the antitumor activity may be due to host mediated events and found IC201 acts on macrophages. The administration of IC201 stimulated phagocytosis and PMA-elicited superoxide production of peritoneal macrophages in mice. In *in vitro* experiments, IC201 did not show any stimulatory effects on MLCR in macrophage-depleted cultures, whereas the addition of IC201 to cultures containing macrophages stimulated it. Moreover, IC201 enhanced production of IL-1 by $P388D_1$ cell cultures. In this experiment, however, IC201 affected stimulatory effect of LPS on IL-1 production. It suggests that IC201 may act on same site(s) of $P388D_1$ cells as where LPS acts. These results indicate that IC201 primarily activates macrophages to induce production of monokines and the activation may modulate immune responses. As mentioned above, IC201 acts on non-stimulated macrophages and may differentiate them into primed-macrophages[10]. These results indicate that IC201 is a low molecular weight immunomodulator and exhibits antitumor effect against a solid tumor through activation of host mediated events. The mechanisms of action are now under study.

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