METHOD OF SEARCH FOR MICROBIAL INHIBITORS OF MEVALONATE BIOSYNTHESIS USING ANIMAL CELLS

HIDETOSHI KUMAGAI, HIROSHI TOMODA and SATOSHI ŌMURA*

The Kitasato Institute and School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan

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A new screening method for specific inhibitors of mevalonate biosynthesis was established using Vero cells, an animal cell line. The cultures selected were those which inhibited the growth of Vero cells in the EAGLE's minimum essential medium supplemented with 2% calf serum (2% CS-MEM) but lacked inhibitory activity against the growth of cells in 2% CS-MEM supplemented with 1 mM mevalonate. By this screening method, inhibitors of the two enzymes involved in mevalonate biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase, were selected from about 11,000 soil isolates. The β-lactone 1233A, a fungal metabolite, was found to be the first naturally occurring compound which inhibits HMG-CoA synthase specifically and strongly. Monacolins K and J, inhibitors of HMG-CoA reductase, were also detected and identified.

Epidemiologic data revealed the surprising fact that more than half of the people in Western industrialized societies have a high level of circulating low density lipoproteins (LDL), particles carrying cholesterol that put them at a high risk of developing atherosclerosis. BROWN and GOLDSTEIN[1] demonstrated the mechanism by which the LDL, receptors influence cholesterol and artherosclerosis. Much attention has been paid to inhibitors of cholesterol biosynthesis as potential hypocholesterolemic agents since these evidences were presented. The fungal metabolites, compactin (ML-236B)[2,3] and mevinolin (monacolin K),[4,5] were discovered as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. Recently, their analogs, pravastatin (CS-514)[6] and simvastatin, have been developed and approved as clinical drugs.

Mevalonate is a key intermediate in cholesterol biosynthetic pathway. It is produced from acetyl-CoA by three enzymes, namely, acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA reductase. These enzymes are expected to provide promising target sites for the pharmaceutical intervention of hypocholesterolemic agents.

We have been developing screening systems according to our strategy of using animal cells or microorganisms, instead of using the relevant enzymes themselves for the discovery of enzyme inhibitors.[7,8] KANEKO *et al.*[9] reported that the growth of cultured animal cells was inhibited by compactin and that the inhibition was overcome by the addition of mevalonate to the medium. It has been thought that most microorganisms cannot incorporate mevalonate into their cells. Therefore, cultured animal cells are expected to be an ideal test organism to discover inhibitors of mevalonate biosynthesis.

In this report we describe the primary screening method for inhibitors of mevalonate biosynthesis using Vero cells, and the inhibitors discovered from about 11,000 soil microorganisms by this screening program.

Materials and Methods

Cell Culture

Vero cells, an established cell line from kidney cells of African green monkey, were used as test organism. The cells were routinely grown in a humidified incubator (95% air/5% CO_2) at 37°C in a 1-liter flask containing 200 ml of EAGLE's minimum essential medium (Gibco Co.) supplemented with benzylpenicillin (50 U/ml), streptomycin (50 μg/ml) and calf serum (Gibco Co.) at 5% (5% CS-MEM). Cells were subcultured according to the conventional trypsinization procedures.

Measurement of Cell Growth

Cell growth was measured by the method of ARMSTRONG.[10] Cells grown on each well of 96-well microplates were washed twice with 100 μl of calcium- and magnesium-free phosphate buffered saline (PBS) and stained with 50 μl of the staining solution (methylrosaniline 0.5%, NaCl 0.85%, formamide 5% and ethanol 50%) for 20 minutes. Then the staining solution was removed and the cells were washed with water. The absorbance at 540 nm was measured by microplate photometer (Titertek Co.)

Screening Method

Vero cells were seeded in each well of a 96-well microplate (Corning Co.) at the concentration of 3×10^4 cells in 100 μl of 2% CS-MEM. After a 1-hour incubation, paper disks (6 mm i.d., Toyo Roshi Co.) each containing microbial cultured broths (50% ethanol solution) were put into the wells. After a 24-hour incubation paper disks were removed. The cell growth was examined with a microscope or microplate photometer by the method as described above if necessary. In the primary selection cultured broths showing growth inhibition of Vero cells were picked up. The secondary selection was carried out as follows. Two sets of paper disks, one set containing various amounts of a sample alone and the other containing various amounts of a sample and 1 mM mevalonate, were put into the wells according to the method as described above. Cultures whose inhibition of Vero cell growth was reversed by the addition of mevalonate were picked up.

Chemicals

DL-Mevalonolactone was purchased from Sigma Co. DL-Mevalonolactone was converted to DL-mevalonate by treatment with 0.1 M NaOH at 60°C for 10 minutes prior to use. Mevalonate and mevalonolactone were equally effective as reversant of this assay. Methylrosaniline chloride (gentian violet B) was purchased from Kanto Chemical Co. Compactin was a generous gift of Dr. A. ENDO, Tokyo Noko University, Tokyo. Other materials were commercially available.

Results

Rationale for the Screening Method

Concentration of Cells: Vero cells were seeded in each well of a 96-well microplate at $1.0 \sim 6.0 \times 10^4$ cells in 100 μl of 2% CS-MEM. Confluent growth was observed after a 24-hour incubation when the cells were seeded at $3.0 \sim 4.0 \times 10^4$ cells/well. Thus, the concentration of 3.0×10^4 cells/well was adopted in subsequent experiments.

Concentration of Mevalonate: When Vero cells (3.0×10^4 cells/100 μl of 2% CS-MEM/well) were incubated with 3 μM (1.2 μg/ml) of compactin for 24 hours, the cell growth was inhibited completely. Further addition of 1 mM mevalonate to the compactin-containing medium did overcome the growth inhibition completely. But the growth was not recovered by the addition of 0.01 mM mevalonate, and was recovered only moderately by 0.1 mM mevalonate. Therefore, 1 mM mevalonate was adopted as a reversant in subsequent experiments.

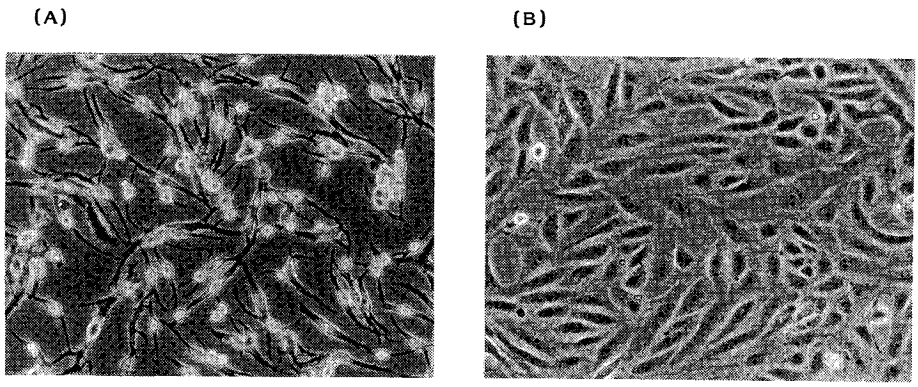
Effect of Various known Compounds in this Screening: Effects of 16 kinds of bioactive compounds on the growth of Vero cells were tested (Table 1). All the compounds, except compactin and fatty acids, showed

Table 1. Effect of various compounds on Vero cell growth with or without mevalonate.

Compound	MIC (μg/ml)		Compound	MIC (μg/ml)	
	None	+Mevalonate (1 mM)		None	+Mevalonate (1 mM)
Amphotericin B	<5.0	<5.0	Nonactin	<5.0	<5.0
Antimycin A	<5.0	<5.0	Nystatin	<5.0	<5.0
Bleomycin	<5.0	<5.0	Oligomycin	<5.0	<5.0
Clindamycin	<5.0	<5.0	Siccanin	<5.0	<5.0
Colistin	<5.0	<5.0	Streptovaricin	<5.0	<5.0
Cerulenin	<5.0	<5.0	Valinomycin	<5.0	<5.0
Doxorubicin	<5.0	<5.0	Compactin	0.1	50
Mitomicin C	<5.0	<5.0	Arachidonic acid	50	50
Monensin	<5.0	<5.0	Linoleic acid	>50	>50
Nanaomycin D	<5.0	<5.0			

Fig. 1. Phase-contrast microscopic appearance of Vero cells incubated for 24 hours with 0.40 μM compactin alone, and in combination with 1 mM mevalonate.

(A) 0.40 μM compactin, (B) 0.40 μM compactin + 1 mM mevalonate.



inhibitory activity against Vero cell growth at 5 μg/ml. The severe growth inhibition caused by 0.1 μg/ml compactin was reversed by the addition of 1 mM mevalonate, but that caused by the other compounds was not reversed. Therefore, inhibitors specifically affecting mevalonate biosynthesis are expected to be positive in this screen. Linoleate did not show any effect on Vero cell growth even at the concentration of 50 μg/ml (Table 1).

Effect of Compactin on Vero Cell Growth

Vero cells were incubated for 24 hours with various concentrations of compactin alone and in combination with 1 mM mevalonate and the cell growth was observed microscopically and measured at 540 nm after staining. Morphological changes of Vero cells were observed at 0.25 ~ 1.0 μM compactin (Fig. 1(A)). When 1 mM mevalonate was added to the medium, the morphological changes disappeared and the cells resumed normal growth (Fig. 1(B)). As shown in Fig. 2, cell growth was slightly inhibited by compactin even at 0.1 μM, and a strong growth inhibition was observed at compactin concentrations higher than 1.0 μM. However, further addition of 1 mM mevalonate permitted the cells to grow normally over the wide range of concentrations of compactin tested. These data indicated that the screening method is highly sensitive to mevalonate biosynthesis inhibitors.

Results of the Screening Run

Broth filtrates of about 10,000 strains of actinomycetes and 1,000 strains of fungal soil isolates were submitted to this screening program. About 30% cultures passed the first selection and only 4 cultures of fungi passed the second selection. All the active products were identified structurally as known compounds, 1233A and monacolins K and J (Fig. 3). That antibiotic 1233A is an inhibitor of mevalonate biosynthesis is a new finding. This required further study.

Culture broth of the fungus *Scopulariopsis* sp. F-244 showed the same activity as compactin

Fig. 2. Effect of compactin alone and in combination with 1 mM mevalonate on Vero cell growth.

○ Compactin, ● compactin + 1 mM mevalonate.

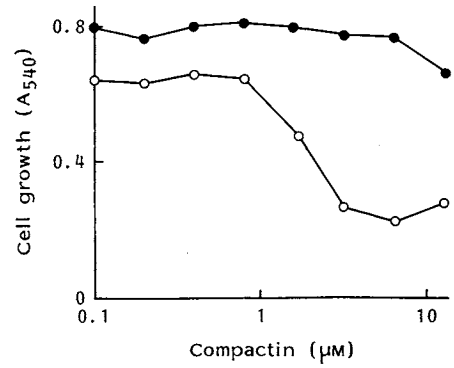
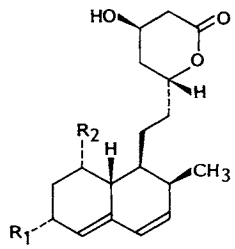
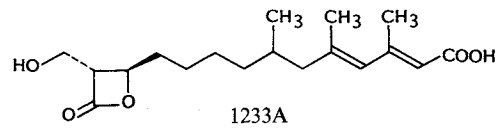
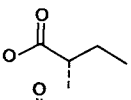


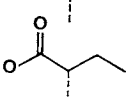
Fig. 3. Structures of compactin, monacolins K and J and 1233A[22].





1233A

Compactin $R_1 = H$ $R_2 =$ 

Monacolin K $R_1 = CH_3$ $R_2 =$ 

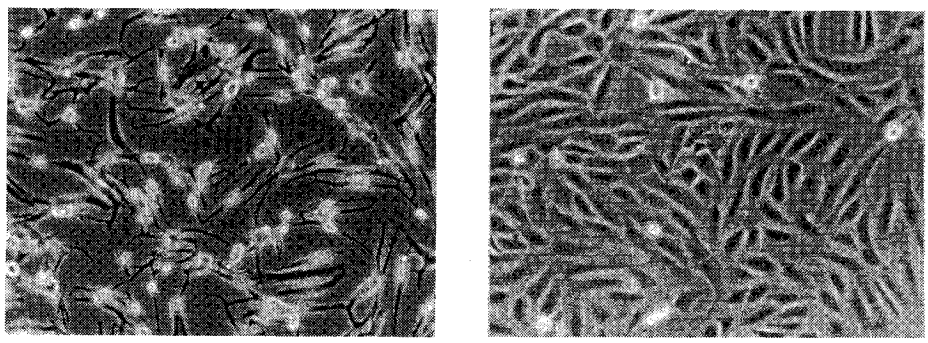
Monacolin J $R_1 = CH_3$ $R_2 = OH$

The absolute configuration was reported by CHIANG *et al.*[22].

Fig. 4. Phase-contrast microscopic appearance of Vero cells incubated for 24 hours with a cultured broth of *Scopulariopsis* sp. F-244 alone and in combination with 1 mM mevalonate.

(A) *Scopulariopsis* sp. F-244, (B) *Scopulariopsis* sp. F-244 + 1 mM mevalonate.

(A) (B)



(Fig. 1) on Vero cells upon the addition of 1 mM mevalonate (Fig. 4). A β-lactone of 1233A was isolated, which was originally reported as an antibiotic by ALDRIDGE *et al.*,[11,12] but no biological studies had been reported. Our screening showed that 1233A inhibits mevalonate biosynthesis. Further studies on the mechanism of action revealed that 1233A is a specific and potent inhibitor of HMG-CoA synthase.[13~15]

The other three strains were found to produce monacolins K and J (Fig. 3), inhibitors of HMG-CoA reductase.[5,16]

Discussion

A number of screening programs for enzyme inhibitors have been extensively carried out by using target enzymes or cell-free enzyme systems as a primary screen. We have developed screening systems for the discovery of microbial enzyme ihibitors by using animal cells or peculiar microorganisms having special functions.[7,8,17] It is expected that this strategy, *i.e.* the cell assay, would be advantageous over the conventional *in vitro* enzyme assay for a primary screening system for the following reasons: 1) The intracellular environment where an enzyme exists is maintained. It would be important to keep the enzyme environments as they are in living cells for screening enzyme inhibitors. 2) Primary assays can be done without radioactive substrates. 3) False positive substances which are active *in vitro* and inactive *in vivo* can be eliminated. It is a problem whether or not an enzyme inhibitor can reach the place where an enzyme reaction takes place. Inhibitors which cannot penetrate membrane barriers or are inactivated by some enzymes might be eliminated. 4) A pro-drug type of inhibitors which is activated after being incorporated into living cells might be discovered. 5) An inhibitor selected by the cell assay would provide higher possibility of *in vivo* efficacy, because a cell assay lies in the middle position between an *in vivo* assay and an *in vitro* assay. On the other hand, some disadvantages also exist. To culture cells is rather troublesome and the running cost is high for routine screening work. It will take more time to evaluate the inhibitory potency by this method. So, unstable inhibitors might be overlooked.

By the screening program for inhibitors of mevalonate biosynthesis described here, only four stains of fungi among 11,000 soil isolates were selected. All the active principles were identified as shown in Fig. 3. KURODA and ENDO[18] reported several long chain fatty acids inhibited cholesterol synthesis *in vitro*. These include tridecanoate, which inhibits acetoacetyl-CoA thiolase, highly unsaturated fatty acids, e.g. arachidonate and linoleate, which inhibit HMG-CoA synthase, and ricinolate and phytanate, which diminish the conversion of mevalonate to sterol. However, none of them were detected during the screening. In fact, the inhibition of Vero cell growth by arachidonate was not reversed by mevalonate, and linoleate showed no effect on Vero cell growth (Table 1), indicating the "noise" due to these compounds could be eliminated in this method. Recently, a novel antifugal antibiotic L-660,631 (Sch 31828) was isolated from actinomycetes[19] and *Microbispora* sp. [20] The antibiotic was found to inhibit acetoacetyl-CoA thiolase.[21] It will be interesting to see the effect of the antibiotic on the growth of Vero cells.

Taken together, our new screening method using animal cells has proved useful in search for new inhibitors of mevalonate biosynthesis. Furthermore, it is expected that this strategy would be extended to the discovery of other types of enzyme inhibitors in the field of screening research.[17]

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