

ELEVATION OF LOW DENSITY
LIPOPROTEIN-RECEPTOR mRNA
CONCENTRATION IN HUMAN HEPATOMA
HepG2 CELLS BY MACROLIDE
ANTIBIOTICS

MASAMI OGASAWARA, NOBUAKI NARUSE,
ATSUKO YOSHIMURA, YASUTARO HAMAGISHI
and TOSHIKAZU OKI

Bristol-Myers Squibb Research Institute,
2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

(Received for publication November 24, 1992)

Plasma cholesterol which is one of risk factors for the development of coronary heart disease is derived both from dietary fat and *de novo* synthesis by the liver¹. There are two main therapeutical approaches to lower plasma cholesterol. The first is to stimulate removal of cholesterol by using an ion exchanger like cholestyramine, which absorbs bile acids synthesized from cholesterol and results in excretion of it from the body as an ion complex. The second is a newer approach to use inhibitory effect on cholesterol biosynthesis of the liver because it is a major site of lipoprotein synthesis. In this pathway, 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a rate-limiting enzyme to convert HMG-CoA to mevalonate. As a function of this enzyme is inhibited, incorporation of cholesterol by the hepatocyte is increased by upregulation of low density lipoprotein-receptor (LDL-R). Compactin was discovered first as HMG-CoA reductase inhibitor from culture broth of fungi². There were also effective agents including pravastatin, lovastatin, simvastatin and fluvastatin, which were developed by modification of compactin to produce more effective derivatives². In the course of our screen for LDL-R upregulators, we found that PC-766B, bafilomycin (16-membered macrolide antibiotics) and concanamycin (18-membered macrolide ones) are potent to increase LDL-R mRNA concentration in human hepatoma HepG2 cells.

To screen LDL-R upregulators in culture broths of microbes, LDL-R mRNA concentration was used as an index, and determined by the Northern blot analysis. Experimental protocol is described in the legend of Fig. 1. During this screen in our laboratory, we found that major active component

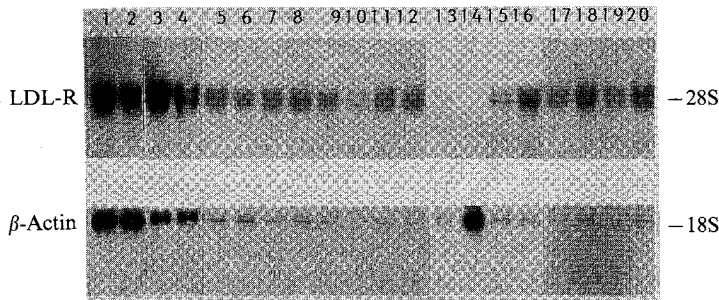
from an unidentified strain of Actinomycetes was identified with PC-766B, which had been already isolated from *Nocardia* sp. (SC-4710) by Sumitomo Pharmaceutical Co. Ltd. and the compound had been reported as a bactericidal antibiotic to Gram-positive bacteria³. We also discovered that other macrolide concanamycin, bafilomycin A1 and C1 were active. The effects of these compounds were demonstrated in Fig. 1. It indicated that concanamycin and bafilomycin C1 at 0.4 $\mu\text{g/ml}$ showed potent LDL-R upregulation activities (3.7 and 6.0 vs. control in upregulation index of LDL-R mRNA; UI_m, respectively) while fluvastatin (positive compound, synthesized by W. E. HARTE in Bristol-Myers Squibb Company), 2.7 vs. control in UI_m at 0.4 $\mu\text{g/ml}$. The activity of PC-766B (3.1 vs. control in UI_m) is close to that of fluvastatin. During purification of PC-766B, another active fraction was isolated as a minor component. This fraction showed 7.0 in UI at 2 $\mu\text{g/ml}$ as LDL-R mRNA upregulation activity when fluvastatin showed 3.6 in UI at 0.4 $\mu\text{g/ml}$.

As to antibacterial activities of the 16- or 18-membered macrolides used here, although we did not investigate their bactericidal activities in detail, the purified material in our laboratory, PC-766B, was active against *Saccharomyces cerevisiae* at concentration below 0.0625 $\mu\text{g/ml}$ and *Candida albicans* at 0.78 $\mu\text{g/ml}$ as minimum inhibitory concentration (MIC) while not active against *Staphylococcus aureus* or *Bacillus subtilis* (up to 0.5 $\mu\text{g/ml}$). The literature values of MIC were obtained from several laboratories; PC-766B and bafilomycins showed 1~13 $\mu\text{g/ml}$ and 9~40 $\mu\text{g/ml}$ as MIC values against Gram-positive bacteria, fungi and yeast^{3,4} while concanamycins exhibited 1~3 $\mu\text{g/ml}$ as MIC values against fungi⁵. It seems that there is not clear relationship between antibacterial and LDL-R upregulation activities of these macrolide antibiotics.

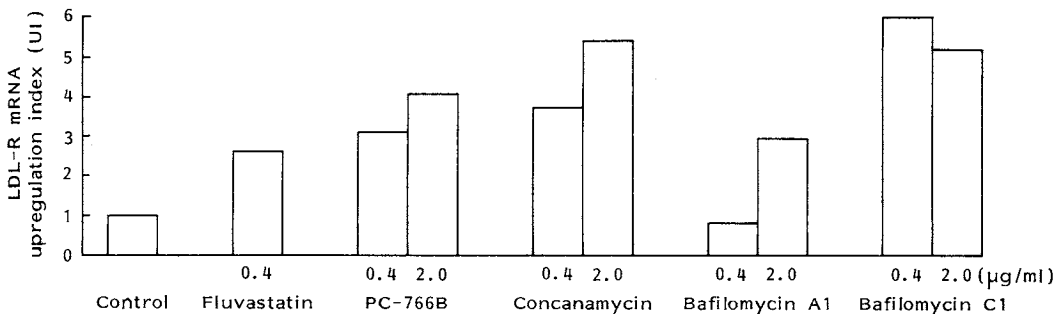
To examine whether elevated concentration of LDL-R mRNA by the compounds is due to stimulation of transcription of LDL-R gene or decrease of LDL-R mRNA degradation, the effects of these compounds on the expression of LDL-R promoter were further confirmed by the promoter assay using LDL-R promoter gene-transfected HepG2 cells as tester cells. These cells were transfected with a DNA plasmid construct containing the promoter of LDL-R linked to an easily

Fig. 1. The effects of PC-766B, concanamycin, bafilomycin A1 and C1 on the concentration of cellular LDL-R mRNA in HepG2 cells.

(A) Autoradiogram



(B) Densitometric determination



HepG2 (5×10^6 , a gift of Bristol-Myers Squibb Institute at Wallingford, CT) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum for 3 days using a collagen-coated plate (Corning, NY). The plates were washed and 3 ml of RPMI1640 (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 2% lipoprotein deficient human serum (LPDS, Sigma Chemical Co., MO) was added. The cells were incubated with or without test samples for 20 hours at 37°C in a humidified atmosphere of 5% CO₂ incubator. Whole RNA (180 μg) was extracted according to the method of CHOMCZYNSKY and SACCHI⁸⁾. Northern blot analysis was carried out by the method of SAMBROOK *et al.*⁹⁾. Cellular RNA (for LDL-R mRNA: about one sixth of whole RNA preparation, β-actin mRNA: 1/100 of that) was separated by formaldehyde (2.2M)-denatured 1% agarose gel, blotted onto a nylon membrane (Nytran-N, Schleicher and Schuell, Dassel, Fed. Rep. of Germany) and hybridized with a ³²P-labeled cDNA probes ($1 \sim 5 \times 10^6$ cpm/ml solution for hybridization); for LDL-R: human LDL-R cDNA (the *Xba*I-*Eco*RI digestion of pUC LDLR4), was a gift of Dr. T. YAMAMOTO (Tohoku University), and exon18 of LDL-R gene (0.7 kb) prepared by *Bam*HI-*Sma*I digestion of pUC19 LDL, for β-actin: 2.1 kb fragment obtained from *Bam*HI digestion of LK221. ³²P-Labeling for the probes was carried out by a random primed DNA labeling kit purchased from Boehringer Mannheim. Following hybridization, the filters was washed, and subjected to autoradiography. Autoradiogram (Panel A) was monitored to determine the relative amounts of mRNA by densitometric scanning after exposure of the filter to Kodak XAR-5; each lane is as follows; control (lane 1 and 2), fluvastatin (lane 3 and 4; $1 \mu\text{M} \equiv 0.4 \mu\text{g/ml}$), PC-766B (lane 5 and 6; $0.4 \mu\text{g/ml}$, lane 7 and 8; $2 \mu\text{g/ml}$), concanamycin (lane 9 and 10; $0.4 \mu\text{g/ml}$, lane 11 and 12; $2 \mu\text{g/ml}$), bafilomycin A1 (lane 13 and 14; $0.4 \mu\text{g/ml}$, lane 15 and 16; $2 \mu\text{g/ml}$), bafilomycin C1 (lane 17 and 18; $0.4 \mu\text{g/ml}$, lane 19 and 20; $2 \mu\text{g/ml}$). The peak areas for LDL-R mRNA or the peak height for β-actin mRNA used as internal control were measured. A ratio of LDL-R mRNA to β-actin's was calculated, and upregulation index (UI) was determined by dividing of the ratio of test sample by that of control. The data represent the means of the duplicate determinations (Panel B).

assayed reporter gene; in this case a gene of human placental alkaline phosphatase was used. The obtained results are briefly presented as follows; 1.6 upregulation index of promoter activity (UIp) vs.

control by PC-766B, 2.3 UIp by concanamycin, 2.1 UIp by bafilomycin A1, 1.6 UIp by bafilomycin C1 and 2.2 UIp by fluvastatin. All concentrations of the compounds tested were $5 \mu\text{g/ml}$ except

fluvastatin (0.4 $\mu\text{g/ml}$). These data suggested that macrolide antibiotics could stimulate expression of promoter of LDL-R; transcription of LDL-R gene was increased and resulted in higher concentration of LDL-R mRNA in HepG2 cells. Therefore, it is suggested that 16- or 18-membered macrolide antibiotics have effects on the expression of LDL-R *via* increase of the expression of promoter gene of LDL-R although it is not clear by our results that the macrolide antibiotics increase the receptor number of LDL. It remains to be resolved.

We can not describe a relationship between the structures and LDL-R upregulator activity because we did not test the activities of other 14- and 15-membered macrolide antibiotics in LDL-R mRNA upregulator activity. Furthermore, it has not been reported that commercially available 16-membered macrolide antibiotics LDL-R upregulator activity including the increase of mRNA and receptor activity. Therefore, this note is a first report which macrolide antibiotics exerted a certain effect on LDL-R upregulation.

It is recently reported that concanamycin B and bafilomycin B1 inhibit hydrolysis of oxidized LDL in lysosome and recycling of its receptor in mouse macrophage cell line^{6,7}. It is likely that macrolide antibiotics have effect on LDL metabolism intracellularly and influence on LDL-R expression.

References

- 1) BROWN, M. S. & J. L. GOLDSTEIN: A receptor-mediated pathway for cholesterol homeostasis. *Science* 232: 34~47, 1986
- 2) KATHAWALA, F. G.: HMG-CoA Reductase inhibitors: an exciting development in the treatment of hyperlipoproteinemia. *Med. Res. Rev.* 11: 121~146, 1991
- 3) KUMATANI, K.; K. TAYA, S. TANAKA, K. MORIGUCHI, M. FUKAZAWA & A. FUKUI (Sumitomo): Antibiotic and protection thereof. *Jpn. Kokai* 5990 ('87), Jan. 12, 1987
- 4) WERNER, G.; H. HAGENMAIER, H. DRAUTZ, A. BAUMGARTNER & H. ZÄHNER: Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity. *J. Antibiotics* 37: 110~117, 1984
- 5) WESTLEY, J. W.; C.-M. LIU, L. H. SELLO, R. H. EVANS, N. TROUPE, J. F. BLOUNT, A. M. CHIU, L. J. TODARO & P. A. MILLER: The structure and absolute configuration of the 18-membered macrolide lactone antibiotic X-4357B (concanamycin A). *J. Antibiotics* 37: 1738~1740, 1984
- 6) WOO, J. T.; C. SHINOHARA, H. YOSHIDA, K. SAKAI, K. HASUMI & A. ENDO: Inhibitors for cholesterol metabolism in macrophage. *Nippon Nogekagaku Kaishi* 65: 365, 2La11, 1991 (in Japanese)
- 7) NAGANUMA, S.; N. KUZUYA, K. SAKAI, K. HASUMI & A. ENDO: Inhibition of the accumulation of lipid droplets in macrophage J774 by bafilomycin B1 and destruxin E. *Biochem. Biophys. Acta* 1126: 41~48, 1992
- 8) CHOMCZYNSKY, P. & N. SACCHI: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156~159, 1987
- 9) SAMBROOK, J.; E. F. FITSCH & T. MANIATIS (*Ed.*): *Molecular Cloning-A Laboratory Manual*. 2nd Ed. Cold Spring Harbor Laboratory Press, 1989