Action of the Peptide Antibiotic Leucinostatin

KIMIKO ISHIGURO* AND TADASHI ARAI

Division of Chemotherapy, Chiba Cancer Center Research Institute, Nitona, Chiba, Japan

Received for publication 19 January 1976

Leucinostatin, an antimicrobial and antitumor antibiotic, was found to be cytotoxic to the murine leukemic cell line, L 1210. A complete inhibition of cell growth was achieved with 0.5 μg of the drug per ml. Leucinostatin showed membrane damage against L 1210 cells as well as mouse erythrocytes. L 1210 cells were partially protected from membrane damage by the addition of glucose to the medium. In contrast, no protective effect of glucose was observed with drug-induced hemolysis. The possible involvement of membrane lipid in the mechanism of action of leucinostatin is suggested by its action on liposomes. Liposomes prepared with lecithin, cholesterol, and an amphiphatic molecule, either dicetyl phosphate or stearylamine, were sensitive to leucinostatin and response to the drug was enhanced as the cholesterol level of liposomes was decreased. Changes in the net charge of the membrane did not show significant effects on leucinostatin action. In the presence of the antibiotic, on the other hand, protein synthesis in cells was markedly inhibited under conditions where the uptake of amino acids into an acid-soluble pool was not affected. However, leucinostatin did not exhibit any inhibitory effect on protein synthesis in a cell-free system from L 1210 cells. It is suggested that the inhibition of protein synthesis results from the initial interaction of the drug with membrane phospholipid.

The activity of polyene macrolides currently in use as antimycotics is mostly limited toward a well-defined group of fungi. On the other hand, several compounds recently developed, such as clotrimazole (16), miconazole, and other derivatives of 1-phenethylimidazole (4), possess a broad spectrum of activity against fungi as well as bacteria. Furthermore, these compounds show a selective toxicity in vivo as chemotherapeutic agents against pathogenic fungi. We have been interested in the mechanism of broad-spectrum activity characteristic of these antimycotic agents. Leucinostatin, which was isolated by Arai et al. in 1973 (1), was chosen as a model compound for the study of action mechanism because of its broad-spectrum activity not only on microorganisms but also for tumor cells.

In this report are described our experimental results with leucinostatin which suggest the interaction of the antibiotic with membrane phospholipid as studied mainly with an artificial membrane (liposome) system.

MATERIALS AND METHODS

Materials. Leucinostatin was prepared from the culture filtrate of *Penicillium lilacinum* as described in a previous paper (1). The antibiotic was dissolved in either ethanol or methanol. Final concentrations of these solvents in the reaction mixture were 0.4%

for ethanol and 0.5% for methanol, at which concentrations none of the effects of these solvents were observed in any of the assay systems.

Dicetyl phosphate was purchased from K & K Laboratories Inc., Plainview, N.Y., and stearylamine was purchased from Wako Pure Chemical Industries, Ltd., Tokyo. Cholesterol was obtained from Nakarai Chemicals, Ltd., Kyoto. All isotopically labeled compounds were purchased from Daiichi Pure Chemical Co., Ltd., Tokyo.

Cells. All experiments with intact cells were performed with a line of L 1210 cells that was kindly supplied by Bono (National Cancer Institute, Bethesda, Md.). Cells were maintained in suspension culture with Eagle minimum essential medium (MEM), supplemented with 10% fetal calf serum. For the cell growth study, cells were suspended at a concentration of 6×10^4 cells/ml and incubated at 37 C for 1 day to allow an exponential phase of growth.

Trypan blue uptake. L 1210 cells (1×10^6 cells/ml) suspended in Dulbecco modified phosphate-buffered saline (PBS) and supplemented with each ingredient of MEM were incubated at 37 C for 4 h with varying concentrations of the drug. Each medium was also supplemented with 10% fetal calf serum. The cell suspension finally received an equal volume of 0.5% dye solution. The mixture was dispersed with a Pasteur pipette and a drop of the suspension was placed on a hemocytometer. Four minutes after dispersing cells, the numbers of stained and unstained cells in a given area on the hemocytometer were counted; usually about 200 cells were counted.

The percentage of stained cells was calculated, i.e., stained cells/(unstained + stained cells) $\times$ 100 (13).

Measurement of hemolysis. The method of Hsuchen and Feingold (6) was slightly modified as follows. Freshly drawn mouse erythrocytes were washed with PBS until the supernatant became clear. They were then diluted into 10 volumes of PBS, and 0.05 ml of this suspension was added to tubes containing leucinos-tatin, at various concentrations, in the presence (1 mg/ml) or in the absence of glucose. The final volume of each sample was adjusted to 1.0 ml with PBS. Incubation was carried out at 37 C for 4 h. The samples were then centrifuged and the extent of hemolysis was determined by the measurement of the absorbancy of the supernatant at 540 nm. The 100% hemolysis value was obtained by diluting of 0.05 ml of cell suspension into 0.95 ml of distilled water, which yielded an absorbancy of 1.178 at 540 nm.

Preparation of liposomes. Liposomes were prepared from egg yolk lecithin, cholesterol, and either dicetyl phosphate or stearylamine in the desired molar ratio by the method of Inoue (7), which is a slight modification of the original method of Kinsky et al. (9). Egg yolk lecithin was prepared in our laboratory by chromatography on aluminum oxide neutral and Unicil. The phospholipid was checked for purity by chromatography on silica gel thin-layer plates with chloroform-methanol-water (130:50:8, vol/vol/vol) as the solvent system. The concentration of the phospholipid was determined by the method of Bartlett (2) for total phosphate. Assays for the permeation rates of glucose, which was trapped as a marker within liposomes, were performed by the method described in reference 9. The total trapped glucose was released by adding Triton X-100 (9).

Macromolecular synthesis in L 1210 cells. Cells used in uptake studies were in the logarithmic phase of growth. Cells (5×10^6 /ml) were harvested in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered MEM supplemented with 5% fetal calf serum and incubated simultaneously with leucinos-tatin and labeled precursors (14 C-labeled amino acids, 0.2 μ Ci/ml; and [3 H]uridine, 0.1 μ Ci/ml). Aliquots of 0.1 ml were taken and the incorporation of the radioactivity into an acid-insoluble fraction was determined by the method of Mans and Novelli (12) except that the degradation of aminoacyl-transfer ribonucleic acid (RNA) was omitted.

Amino acids and uridine influx. The procedure that has been reported by several groups (3, 14, 15) was followed. L 1210 cells, suspended to a concentration of 5×10^6 cells/ml in HEPES-buffered MEM supplemented with 5% fetal calf serum with varying concentrations of leucinos-tatin, were labeled with both 14 C-labeled amino acids (0.2 μ Ci/ml) and [3 H]uridine (0.1 μ Ci/ml) at 37 C. At the times indicated, the influx was stopped by addition of ice-cold PBS. The cells were then extracted with 5% trichloroacetic acid and the extracts were neutralized. After the addition of Bray's solution, the radioactivities were determined with a liquid scintillation counter.

RESULTS

Effect of leucinos-tatin on L 1210 cell growth in culture. Figure 1 illustrates the inhibitory effect of leucinos-tatin on the growth of cultures of L 1210 cells. Cells were inoculated at a concentration of 6×10^4 cells/ml and incubated at 37 C for 1 day to allow for an exponential growth. Thereafter, cultures were exposed to different concentrations of leucinos-tatin for 3 days. Leucinos-tatin showed cytotoxicity toward L 1210 cells over a wide range of concentrations tested, and nearly complete growth inhibition was observed at 0.5 μ g/ml or greater.

Effect of leucinos-tatin on membrane damage. As a next approach the effects of this drug on membrane damage of L 1210 cells and on hemolysis of erythrocytes were studied. The results presented in Fig. 2A reveal that leucinos-tatin produces membrane damage against L 1210 cells. Fifty percent of cells in the population were stained with trypan blue at a concentration of 25 μ g/ml in PBS solution, but in MEM the cells were protected from membrane damage caused by this agent; i.e., the concentration of leucinos-tatin required for 50% membrane damage was 75 μ g/ml. This protective component in MEM was identified as glucose. The addition of 2-deoxyglucose, however, did not show any protective effect (data not shown).

The experiments performed with mouse erythrocytes (Fig. 2B) indicated that leucinos-tatin produced 50% release of hemoglobin at a

FIG. 1. Effect of leucinos-tatin on L 1210 cell growth in culture. Exponentially growing L 1210 cells (1×10^5 /ml) were incubated with different concentrations of the drug and cell number was determined twice a day for 3 days.

FIG. 2. (A) Effect of leucinostatin with or without glucose on membrane permeability of L 1210 cells. L 1210 cells were exposed to leucinostatin in PBS supplemented with various ingredients of MEM. Membrane permeability was determined by the uptake of trypan blue as described in Materials and Methods. L 1210 cells suspended in PBS, ○; PBS plus Mg^{2+} and Ca^{2+} , □; PBS plus vitamins, △; PBS plus amino acids, ▽; PBS plus glucose, ▲; PBS plus all components, ■; and MEM, ●. (B) Effect of leucinostatin on hemolysis of mouse erythrocytes. The detailed procedure is described in Materials and Methods. Mouse erythrocytes suspended in PBS (●), and PBS plus glucose (1 mg/ml) (■).

concentration of 100 $\mu\text{g/ml}$, regardless of presence or absence of glucose.

Effect of leucinostatin on liposomes. Because leucinostatin has been found to cause a membrane damage against both L 1210 cells and mouse erythrocytes and also considering its hydrophobic nature, it seemed likely that it might primarily interact with the lipid constituents of the membrane. Therefore, we examined the effect of the antibiotic on the permeability of an artificial membrane system, the liposome.

Liposomes were prepared with egg yolk lecithin, with glucose as a marker trapped within the aqueous region of the liposome. The glucose released from liposomes by leucinostatin was assayed enzymatically. Aliquots of liposome preparations (5 μl) that contained egg lecithin, dicetyl phosphate, and cholesterol in the molar ratio of 1:0.1:1 were incubated with varying concentrations of leucinostatin for 30 min at 22 C. As shown in Fig. 3, leucinostatin drastically enhanced glucose permeability of the liposomes under conditions where the spontaneous release was not significantly affected. At concentrations below 20 $\mu\text{g/ml}$, leucinostatin showed no appreciable effect on liposomes.

In recent years, data have been accumulated that cholesterol, a major lipid constituent of many biological membranes, regulates the fluidity of hydrocarbon chains in the lipid bilayer under physiological conditions (7, 11). We therefore examined whether the susceptibility toward this antibiotic was affected by the level of cholesterol of the liposomes. When lesser

FIG. 3. Time course of glucose release induced by leucinostatin. Liposomes prepared with egg lecithin, dicetyl phosphate, and cholesterol (molar ratio 1:0.1:1) were incubated with varying concentrations of leucinostatin at 22 C. At the times indicated, glucose released from liposomes was assayed enzymatically.

amounts of cholesterol were initially incorporated into liposomes, an increased sensitivity to leucinostatin was demonstrated (Fig. 4). In the case of liposomes prepared with egg lecithin, dicetyl phosphate and cholesterol in the molar ratio 1:0.1:0.125, the reaction was extremely rapid, and complete release of the glucose marker was observed within 2 min. These results suggest that increased fluidity of the membrane resulting from the introduction of low levels of cholesterol into liposomes may enhance the disorganization of the lipid bilayer by leucinostatin.

Experiments were also performed to deter-

FIG. 4. Effect of the level of cholesterol incorporation into liposomes on the extent of glucose released by leucinostatin at 200 $\mu\text{g}/\text{ml}$. Liposomes were prepared with egg lecithin, dicetyl phosphate, and various amounts of cholesterol. The molar ratios of lecithin-dicetyl phosphate-cholesterol were 1:0.1:1, $\bullet$; 1:0.1:0.5, $\blacksquare$; 1:0.1:0.25, $\blacktriangle$; and 1:0.1:0.125, $\blacktriangledown$. Conditions for the incubation of liposomes were as described in the legend of Fig. 3.

mine how the drug sensitivity was influenced by the incorporation of various amounts of amphipathic molecules, such as negatively charged dicetyl phosphate or positively charged stearylamine. The data presented in Fig. 5 show that, in negatively charged membranes, there was little difference in drug sensitivity. In contrast, the incorporation of decreased amounts of stearylamine enhanced glucose permeability caused by leucinostatin (Fig. 5). However, positively charged liposomes that contained egg lecithin, stearylamine, and cholesterol (molar ratio 1:0.1:1) were shown to be damaged to almost the same extent as negatively charged liposomes in which stearylamine was replaced by equimolar dicetyl phosphate. Thus, these results provide an indication that the change in the net charge on liposomal membrane does not play an important role for the interaction of leucinostatin with liposomes.

Effect on macromolecular synthesis and precursor pools. Figure 6A and B illustrates the effect of leucinostatin on the macromolecular synthesis in L 1210 cells in culture. The cells were continuously labeled with radioactive precursors of RNA and protein, and the radioactivities incorporated into the corresponding fractions were measured at the times indicated. At a concentration of 10 $\mu\text{g}/\text{ml}$, significant inhibition of the protein synthesis was observed, while the effect on RNA synthesis was not as remarkable as in the case of the protein synthe-

sis. The synthesis of deoxyribonucleic acid was not affected under the same conditions (data not shown).

In an attempt to understand the mechanism of the inhibition of protein synthesis, the effect of leucinostatin on amino acid uptake was studied. Under conditions where the protein synthesis was inhibited by approximately one-third, the influx of amino acids was barely suppressed (Fig. 7A). The drug did not affect the influx of uridine (Fig. 7B), as expected from its effect on RNA synthesis. These results indicate that the inhibition of protein synthesis was not a result of the inhibition of amino acid transport.

Since leucinostatin markedly inhibited protein synthesis of intact L 1210 cells, its effect on cell-free extracts was investigated (data not shown). However, it did not have any inhibi-

FIG. 5. Effect of the incorporation of amphipathic molecules into liposomes on the extent of glucose released by leucinostatin at 200 $\mu\text{g}/\text{ml}$. Liposomes containing varying amounts of amphipathic molecules were incubated at 22 C for 60 min. Liposomes were prepared from dicetyl phosphate ($\bullet$) or stearylamine ($\blacksquare$).

FIG. 6. Effect of leucinostatin on amino acid and uridine incorporation into acid-insoluble fraction of L 1210 cells. The suspension of L 1210 cells in HEPES-buffered MEM plus 5% fetal calf serum was incubated with labeled precursors at 37 C. Radioactivities of ^{14}C -labeled amino acids (A) and ^3H -uridine (B) were determined at the indicated times. Control ($\bullet$), and leucinostatin (10 $\mu\text{g}/\text{ml}$) ($\blacksquare$).

FIG. 7. Effect of leucinostatin on amino acid and uridine incorporation into acid-soluble fraction of L 1210 cells. Experimental conditions were the same as in Fig. 6. At the times indicated, cells were washed 3 times with PBS and suspended in cold 5% trichloroacetic acid and radioactivities of ¹⁴C-labeled amino acids (A) and [³H]uridine (B) were determined. Control (●), and leucinostatin (■).

tory effect on the incorporation of amino acid into protein fraction directed with either endogenous messenger RNA or poly(U). Thus, there is a discrepancy between the marked inhibition of protein synthesis in intact cells and the lack of an effect on cell-free lysates.

DISCUSSION

Many cyclic or linear peptide antibiotics and polyene antifungals are well known to interact with the cytoplasmic membrane to cause damage and ultimate destruction of the membrane. Some of them also exhibit an inhibitory effect on membrane-bound enzymes involved in either transport or oxidative phosphorylation, or induce changes in the permeability for specific ions. Leucinostatin was shown to cause membrane damage in L 1210 cells as well as hemolysis of erythrocytes. The membrane damage of L 1210 cells was partially prevented by the addition of glucose to the medium. The protection by a metabolizable sugar, but not by a nonmetabolizable one, can be explained by the assumption that this drug might primarily produce a drain on the energy-yielding system. The same assumption could account for the fact that the lysis of erythrocytes, which lack mitochondria, was not protected by the presence of glucose. Similar phenomena have been reported for cytochalasin A with yeast cells by Kuo and Lampen (10) in which the inhibition of uptake of amino acids or uridine by cytochalasin A was reversed by the addition of glucose.

Artificial membranes, liposomes, have extensively contributed to studies of the action mechanism of drugs affecting the cytoplasmic membrane. The fact that leucinostatin induces a permeability change of liposomes suggests that this drug interacts with lipid constituents

of biological membranes. The decrease in the level of cholesterol in liposomes resulted in higher sensitivity to the antibiotic. It was shown that in the case of lysolecithin, a change in the level of cholesterol in liposomes, hence an altered fluidity of membranes, also affected drug sensitivity (8). The action of leucinostatin on liposomes was irrelevant to the change in the net charge of the membranes. The precise mechanisms as to how this drug causes damage in the energy-yielding system through its interactions with lipid components are still unknown.

With an intact cell system, an inhibition of protein synthesis was detected before membrane damage or destruction, i.e., the inhibition of protein synthesis was observed as early as 20 min after the addition of the drug (Fig. 6A), while membrane damage of L 1210 cells was not found even after 4 h (Fig. 2A) at the same drug concentration. However, no inhibition of protein synthesis was demonstrated with a cell-free system. The inhibition of protein synthesis in intact cells could be mediated by disorganization of membranes. Although amino acid influx was not affected by this drug, the damage to transport of other important factor(s) for protein synthesis could perhaps induce an inhibition of protein synthesis. One such factor might be potassium ion, as suggested by Harold and Baarda in the study of gramicidin (5).

Finally, the primary site of action of this antibiotic is thus assumed to be the interaction with membrane phospholipid. This assumption is further substantiated by the fact that leucinostatin is highly active on bacteria that do not contain cholesterol (1).

ACKNOWLEDGMENTS

We wish to thank K. Inoue (Faculty of Pharmaceutical Sciences, Tokyo University, Tokyo, Japan) for helpful suggestions during the course of liposome experiments.

This work was supported by grants-in-aid from the Japanese Education Ministry, Science and Culture, and from the Japan Ministry of Welfare.

LITERATURE CITED

1. Arai, T., Y. Mikami, K. Fukushima, T. Utsumi, and K. Yazawa. 1973. A new antibiotic, leucinostatin, derived from *Penicillium tilacinum*. *J. Antibiot.* 26:157-161.
2. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466-468.
3. Dunhan, P. B., I. M. Goldstein, and G. Weissmann. 1974. Potassium and amino acid transport in human leukocytes exposed to phagocytic stimuli. *J. Cell Biol.* 63:215-226.
4. Godefroi, E. F., J. Heeres, J. Van Cutsem, and P. A. J. Janssen. 1969. The preparation and antimycotic properties of derivatives of 1-phenethylimidazole. *J. Med. Chem.* 12:784-791.
5. Harold, F. M., and J. R. Baarda. 1967. Gramicidin,

- valinomycin, and cation permeability of *Streptococcus faecalis*. *J. Bacteriol.* 94:53-60.
6. Haugen, C., and D. Feingold. 1973. The mechanism of polymyxin B action and selectivity toward biologic membranes. *Biochemistry* 12:2105-2111.
 7. Inoue, K. 1974. Permeability properties of liposomes prepared from dipalmitoyllecithin, dimyristoyllecithin, egg lecithin, rat liver lecithin and beef brain sphingomyelin. *Biochim. Biophys. Acta* 339:390-402.
 8. Inoue, K., and T. Kitagawa. 1974. Effect of exogenous lysolecithin on liposomal membranes its relation to membrane fluidity. *Biochim. Biophys. Acta* 363:361-372.
 9. Kinsky, S. C., J. A. Haxby, D. A. Zopf, C. R. Alving, and C. B. Kinsky. 1969. Complement-dependent damage to liposomes prepared from pure lipids and Forssmann hapten. *Biochemistry* 8:4149-4158.
 10. Kuo, S. C., and J. O. Lampen. 1974. The action of antibiotics on enzyme secretion in yeasts: studies with cytochalasin A. *Ann. N.Y. Acad. Sci.* 235:137-148.
 11. Ladbroke, B. D., R. M. Williams, and D. Chapman. 1968. Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry and X-ray diffraction. *Biochim. Biophys. Acta* 150:333-340.
 12. Mans, R. J., and G. D. Novelli. 1961. Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. *Arch. Biochem. Biophys.* 94:48-53.
 13. Phillips, H. J. 1973. Dye exclusion tests for cell viability, p. 406-408. In F. K. Paul and M. K. Patterson (ed.), *Tissue culture*. Academic Press Inc., New York.
 14. Plagemann, P. G. W., and M. F. Roth. 1969. Permeation as the rate-limiting step in the phosphorylation of uridine and choline and their incorporation into macromolecules by Novikoff hepatoma cells. Competitive inhibition by phenethyl alcohol, persantin and adenosine. *Biochemistry* 8:4782-4789.
 15. Plagemann, P. G. W., G. A. Ward, B. W. Mahy, and M. Korbecki. 1969. Relationship between uridine kinase activity and rate of incorporation of uridine into acid-soluble pool and into RNA during growth cycle of rat hepatoma cells. *J. Cell Physiol.* 73:233-250.
 16. Plempel, M., K. Bartmann, K. H. Bruechel, and E. Regal. 1969. Experimentelle Befunde ueber ein neues, oral wirksames Antimykotikum mit breiten Wirkung-spektrum. *Dtsch. Med. Wochenschr.* 94:1356-1364.