INHIBITION OF PINOCYTOSIS BY HYGROLIDIN FAMILY ANTIBIOTICS: POSSIBLE CORRELATION WITH THEIR SELECTIVE EFFECTS ON ONCOGENE-EXPRESSED CELLS

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A fermentation broth of *Streptomyces* sp. SIPI-A4-0044 inhibited *in vitro* growth of *src* or *ras* oncogene-expressed (onc+) cells more strongly than that of oncogene-unexpressed (onc−) counterparts. The active components were isolated and identified as hygrolidin family antibiotics (HGL). In mixed cultures consisting of onc+ and onc− cells, at an appropriate ratio, HGL showed selective toxicity to focus like structures of onc+ cells, leaving monolayer areas of onc− cells little damaged. HGL rapidly inhibited pinocytosis, or the influx of neutral red into the cells, at concentrations partially inhibitory to the cell growth. In contrast, HGL only slightly inhibited the influx of 2-deoxyglucose, nucleosides and leucine and the syntheses of DNA, RNA and protein even at high concentrations. Upon prolonged exposure to sublethal concentrations of HGL, onc− cells but not onc+ cells recovered pinocytotic activity and resumed growth.

Malfunction of cellular oncogenes is a cause of human cancer[1,2]. From the viewpoint of cancer chemotherapy, it is important to find drugs which are preferentially active against tumor cells where oncogenes are abnormal or abnormally expressed, but are not active against nontumorous counterparts. For this purpose, we used rat kidney cells harboring a temperature sensitive oncogene, either src^{ts} or ras^{ts}, because of the convenient characteristics of the cells shifting between the tumorous and nontumorous state simply by changing culture temperatures. During the course of screening microbial products which possibly inhibit growth of these cells more strongly at 33°C (a permissive temperature) than at 39°C (a nonpermissive temperature), we found a strain of *Streptomyces* the fermentation broth of which showed this activity. The active components were isolated and identified as hygrolidin[3]-bafilomycin[4] family antibiotics, 16-membered macrolides. We further studied the biochemical basis for their selective effects on oncogene-expressed cells and found that they rapidly inhibited pinocytotic activity of the cells at concentrations partially inhibitory to the cell growth. Upon prolonged exposure (24 hours or longer), oncogene-unexpressed cells, as opposed to oncogene-expressed cells, acquired resistance to the antibiotic and resumed growth in parallel with the recovery of pinocytotic activity. This paper reports these results.

Materials and Methods

Cells and Culture Conditions

A rat kidney cell line infected with ts25 mutant of Rous sarcoma virus Prague strain[5] (*src*^{ts}NRK) and another rat kidney cell line infected with ts371 mutant of Kirsten murine sarcoma virus[6] (*ras*^{ts}NRK) were provided by Drs. M. YOSHIDA, Cancer Institute, Tokyo and T. Y. SHIH, NIH, Bethesda, respectively. An NIH/3T3 cell line infected with Rous sarcoma virus SR-D (*src*3T3) was provided by Dr. S. KAWAI, the Institute of Medical Science, University of Tokyo, Tokyo. An uninfected NIH/3T3 cell line was provided by Japanese Cancer Research Resources Bank, Tokyo. Rat kidney cell lines of a fibroblast type (NRK-49F, CRL1570) and of an epithelial type (NRK-52E, CRL1571) and another rat kidney cell line infected with the wild type Kirsten murine sarcoma virus (*ras*NRK, CRL1569) were obtained from American Type Culture Collection. These cells were grown in DULBECCO's modified Eagle medium (DMEM) supplemented with 2~5% heat-inactivated calf serum, in 5% CO_2-containing humidified air at 33°C or 39°C (*src*^{ts}NRK and *ras*^{ts}NRK) or 37°C (the other cell lines). Generally, Costar 12-well tissue culture clusters (4 cm^2/well) or Lux tissue culture dishes (9 cm^2/dish) were used as culture vessels and in each vessel, $2\sim3\times10^4$ cells were seeded with 1.5~2.0 ml medium (day 0). The cells received a test sample on day 1, and were further cultured until day 3~4. Cell growth was quantitated using colorimetric determination of amounts of crystal violet taken up into cell mass[7] or cell counts. Specific conditions are described in legends for figures. Duplicate experiments were run for each set of experimental conditions.

Determination of the Effects of HGL on Influx of Neutral Red (a) and FITC-dextran (b)

(a) Vessels were seeded with *src*^{ts}NRK cells at 10^5 cells/1.5 ml medium/well (4 cm^2) and incubated at 33°C or 39°C. After 20 hours, HGL at various concentrations was added to each well, and 4 minutes later, 75 μl of 0.09% aqueous solution of neutral red was added to each well in the dark, then incubated for another 30 minutes at 33°C. The medium was removed and the cell layers were washed quickly with 1 ml of cold Ca^{2+}- and Mg^{2+}-free PBS/well three times (stained cells). The neutral red in the stained cells was extracted with 2 ml of 50% ethanol-0.1 M NaH_2PO_4/well and absorbancy determined at 540 nm.

(b) The number of cells seeded was 7×10^4 cells/1.5 ml medium/well and the cells were grown and treated with HGL as above. A 50-μl aliquot of FITC-dextran solution in Ca^{2+}- and Mg^{2+}-free PBS at 40 mg/ml was added to each well and incubated at 33°C for another 15 minutes. The cell layers were washed quickly and FITC-dextran taken up into cells was extracted with 1 ml of 20% SDS/well, diluted with 1.5 ml of water/well and quantitated by fluorometry in Hitachi F-4000 with excitation and emission wavelengths set at 485 nm and 515 nm, respectively.

Results and Discussion

Production, Isolation and Identification of the Hygrolidin Family Antibiotics

A fermentation broth of *Streptomyces* sp. SIPI-A4-0044, when assayed against the cultures of *src*^{ts}NRK, inhibited cell growth more strongly at 33°C than at 39°C; for 50% inhibition of cell growth, 2 μl and 16 μl of the broth/2 ml medium/dish were required at the former and the latter temperatures, respectively. Three active components were isolated by a series of purification procedures including Diaion HP-20 column chromatography, extraction with BuOAc-water at pH 2, silica gel column chromatography, HPLC with Nucleosil 50-5, Sephadex LH-20 column chromatography and silica gel TLC. They were tentatively named hygrolidins K_1, K_2 and K_3 because they appeared to share the hygrolidin structure. Hygrolidins K_2 and K_1 were identified as a known compound (L-155,175)[8] and its C19-OCH_3 derivative, respectively, based on NMR and mass spectrometry studies while K_3 has not been identified yet. Biochemical experiments were conducted with K_2.

Selective Effects of HGL on Growth and Viability of Oncogene-expressed Cells

HGL inhibited growth of *src*^{ts}NRK more strongly at 33°C (onc+) than at 39°C (onc−) and the

Fig. 1. Effect of hygrolidin on the growth of *src*^{ts}NRK cells.

Cells were incubated at 33°C (A) or 39°C (B) with or without (●, ○) HGL. HGL was added at day 1 at 0.2 μg/ml (▲, △) or 0.3 μg/ml (■, □).

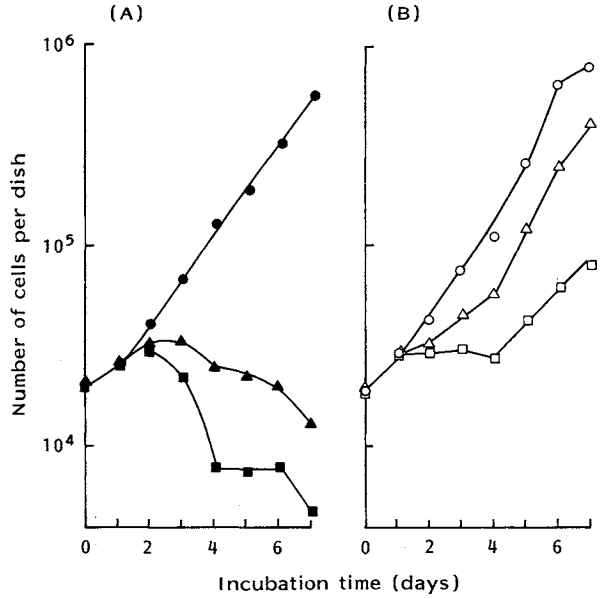
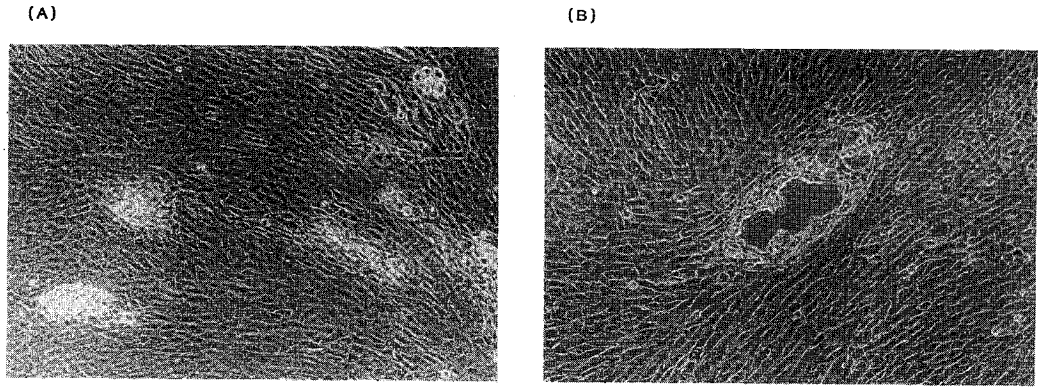


Fig. 2. Phase-contrast micrographs of mixed cultures.

(A) Control, (B) exposed to K_2 at 0.4 μg/ml for 24 hours.



difference in the effects between the two temperatures became more pronounced with longer incubation times (Fig. 1). It should be noted that the cells grown at 39°C, but not at 33°C, gradually acquired resistance to the antibiotic and resumed growth as the incubation was continued. Parallel experiments using *ras*^{ts}NRK gave similar results. To rule out the possibility that the temperature difference, rather than expression or unexpression of the oncogenes, was the cause for the differential effects, additional experiments were conducted using a pair of *src*3T3 (onc+) and NIH3T3 (onc−), and a pair of *ras*NRK (onc+) and NRK-52E (onc−). In either case, the antibiotic inhibited the growth of onc+ cells more strongly than that of onc− cells. For further confirmation, we prepared mixed cultures consisting of *ras*NRK (onc+)

Fig. 3. Effect of hygrolidin on influx of neutral red into *src*^{ts}NRK cells grown at 33°C or 39°C.

Cells were incubated at 33°C (●) or 39°C (○).

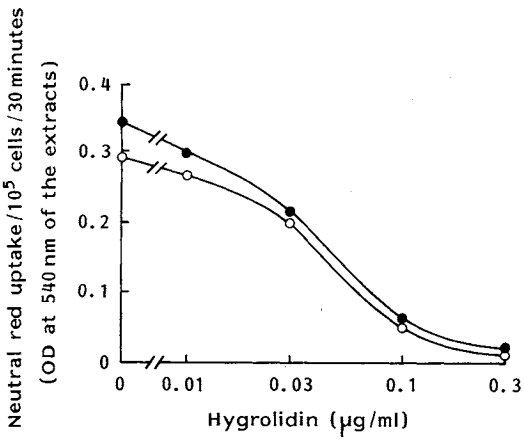
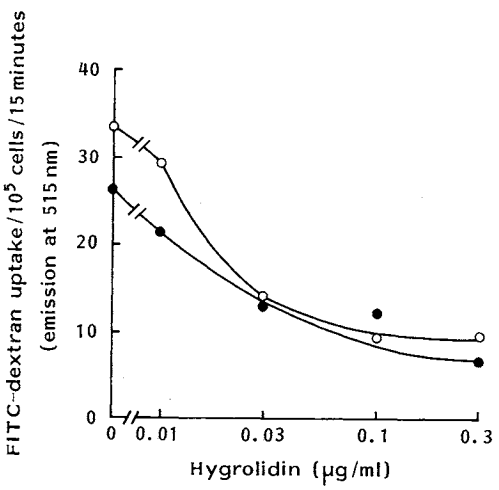


Fig. 4. Effect of hygrolidin on influx of FITC-dextran into *src*^{ts}NRK cells grown at 33°C or 39°C.

Cells were incubated at 33°C (●) or 39°C (○).



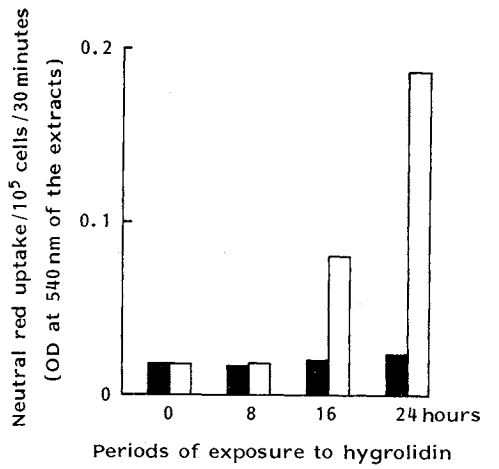
and NRK-49F (onc−) inoculated at 1/1,000 ratio and tested if the antibiotic would damage only focus like structures consisting of *ras*NRK but not monolayer areas consisting of NRK-49F. The results were as we expected (Fig. 2).

Inhibition of Neutral Red Influx by HGL

Among various biochemical indices determined with *src*^{ts}NRK in the presence of HGL, the most rapidly and strongly inhibited was the influx of neutral red, or pinocytosis[9] (Fig. 3). Pinocytosis of FITC-dextran[10] was as sensitive to HGL as was the influx of neutral red (Fig. 4). Other biochemical indices such as influx of labeled 2-deoxyglucose, some nucleosides and leucine into cells and incorporation of labeled thymidine, uridine and leucine, into the acid-insoluble materials of cells were little affected by HGL at concentrations as high as 1.0 μg/ml. It should be noted that the effects on pinocytosis appeared in as early as 30 minutes after addition of HGL at concentrations lower than the growth inhibitory range (Fig. 1 vs. Figs. 3 and 4). The results suggested that major target of HGL was some membrane component(s) playing a role in pinocytosis and that there was no difference in the initial sensitivity of the target to HGL between onc+ cells and onc− cells. This was consistent with the inhibitory effect of HGL on cell growth during the first 24 hours (Fig. 1).

Fig. 5. Neutral red influx into *src*^{ts}NRK cells cultured at 33°C or 39°C in the presence of hygrolidin.

Cells were incubated at 33°C (■) or 39°C (□) with 0.3 μg/ml HGL. At the indicated time, the rate of neutral red influx was measured.



Correlation between Pinocytotic Activity and Cell Growth upon Prolonged Exposure to HGL

Pinocytotic activity of *src*^{ts}NRK cells grown at 39°C (onc−), contrary to those grown at 33°C (onc+),

progressively recovered upon prolonged exposure to HGL (within 24 hours; Fig. 5) resulting in resumption of cell growth with certain periods of delay (2 days; Fig. 1 vs. Fig. 5). The resistance to HGL and the recovery of pinocytotic activity of onc− cells were confirmed by renewing the medium with the fresh one containing the antibiotic at 0.3 μg/ml on day 4. How the pinocytotic activity of onc− cells but not onc+ cells recovers upon prolonged exposure to HGL remains to be elucidated. It is unlikely that onc− cells inactivate HGL more quickly than onc+ cells because the differential effect of HGL on onc+ and onc− cells was also obvious in the mixed cultures (Fig. 2) where both onc+ and onc− cells were in the same culture vessels and exposed to the same concentrations of HGL. The results presented here indicate that expression of some oncogenes alter membrane function(s) involved in pinocytosis and that such alteration could be a target of cancer chemotherapy.

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References

1) DUESBERG, P. H.: Activated proto-onc genes: sufficient or necessary for cancer? Science 228: 669～677, 1985
2) KLEIN, G. & E. KLEIN: Evolution of tumours and the impact of molecular oncology. Nature 315: 190～195, 1985
3) SETO, H.; H. AKAO, K. FURIHATA & N. ŌTAKE: The structure of a new antibiotic, hygrolidin. Tetrahedron Lett. 23: 2667～2670, 1982
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) CHEN, Y. C.; M. J. HAYMAN & P. K. VOGT: Proteins of mammalian cells transformed by temperature-sensitive mutants of avian sarcoma virus. Cell 11: 513～521, 1977
6) SHIH, T. Y.; M. O. WEEKS, H. A. YOUNG & E. M. SCOLNICK: p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. J. Virol. 31: 546～556, 1979
7) SUZUKAKE-TSUCHIYA, K.; Y. MORIYA, K. YAMAZAKI, M. HORI, N. HOSOKAWA, T. SAWA, H. IINUMA, H. NAGANAWA, C. IMADA & M. HAMADA: Screening of antibiotics preferentially active against *ras* oncogene-expressed cells. J. Antibiotics 43: 1489～1496, 1990
8) GOETZ, M. A.; P. A. MCCORMICK, R. L. MONAGHAN, D. A. OSTLIND, O. D. HENSENS, J. M. LIESCH & G. ALBERS-SCHONBERG: L-155,175: A new antiparasitic macrolide. Fermentation, isolation and structure. J. Antibiotics 38: 161～168, 1985
9) HAMMOND, M. E.; J. GOODWIN & H. F. DVORAK: Quantitative measurements of neutral red uptake and excretion by mammalian cells. J. Reticuloendothel. Soc. 27: 337～346, 1980
10) WEISMAN, L. S.; R. BACALLAO & W. WICKNER: Multiple methods of visualizing yeast vacuole permit evaluation of its morphology and inheritance during the cell cycle. J. Cell Biol. 105: 1539～1547, 1987