Vol. 136, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1986 Pages 273-280

EFFECTS OF THE VIBRIO CHOLERAE SIDEROPHORE VIBRIOBACTIN ON THE GROWTH CHARACTERISTICS OF L1210 CELLS

Raymond J. Bergeron[1], Raul Braylan[2], Stacia Goldey[2] and Michael Ingeno[1]

[1]Department of Medicinal Chemistry and
[2]Department of Pathology University of Florida,
Gainesville, Florida 32610

Received February 24, 1986

The microbial iron chelator vibriobactin, N-[3-(2,3- dihydroxybenzamido)propyl]-1,3-bis[2-(2,3-dihydroxyphenyl)-trans-5-methyl-2-oxazoline-4-carboxamido]-propane, is shown to inhibit the growth of L1210 cells in culture, with an IC_{50} value of 2 μM. Its biological activity is assigned to the ligand's ability to chelate iron as indicated by the disappearance of its antimitotic properties on iron chelate preformation or on O-methylation. The ligand is shown to have pronounced effects on cell cycle kinetics introducing a G_1/S phase block. When treated cells are washed free of the ligand with fresh media they cascade into a high S phase in 5 hrs. Furthermore, after exposure of L1210 cells to vibriobactin (10 μM) for 5 hrs followed by removal of the drug, cells display different doubling times relative to untreated controls, with lower bromodeoxyuridine (BrdUrd) incorporation and no apparent cell death as shown by a ^{51}Cr release assay. The effects on cell kinetics are consistent with inhibition of ribonucleotide reductase. Finally, vibriobactin is also shown to be active with a human Burkitt lymphoma cell line (Daudi).

The role of iron in infection and neoplasia has received considerable attention in recent years (1-3). It is not surprising that a metal which serves as a prosthetic group for so many redox enzymes would act as a controlling micronutrient in biological processes.

In keeping with this observation it has been shown that when a patient contracts cancer his serum iron level plummets (2). It has been suggested that this is a defense mechanism which the body utilizes to protect itself against neoplasm. We have in fact successfully simulated this iron withholding status in vitro by exposing L1210 cells to microbial siderophores, microbial iron chelators (4). These compounds, catecholamide iron chelators, bind iron tenaciously, with formation constants in excess of 10^{36} M^{-1}. They were shown to inhibit L1210 cell growth with IC_{50} values in the micromolar range with the most active of the ligands, parabactin, having an IC_{50} value of 2 μM. The activity of these ligands correlated very well with their lipophilicity; the most lipophilic of a group of

0006-291X/86 $1.50

ligands of similar iron binding ability being the most active. Furthermore, there was some suggestion from our initial studies (4) that the chelators' activity is associated with their ability to inhibit the iron dependent enzyme ribonucleotide reductase (5).

More recently, we investigated the mechanism by which parabactin and bis N^1,N^8-(2,3 dehydroxybenzoyl) spermidine (Compound II) inhibit L1210 cell growth (7). Again, in keeping with the siderophores' influence on ribonucleotide reductase, L1210 cells treated with 5 μM parabactin for only 4 hrs accumulated at the G_1/S border. Furthermore, the cells experienced both a decrease in thymidine incorporation and in dATP pools. These findings encouraged us to evaluate the effects of vibriobactin, a highly lipophilic siderophore isolated from Vibrio cholerae, on the growth characteristics of both L1210 cells and Daudi cells. In this study we demonstrate that vibriobactin can serve as a potent non-toxic reversible cell cycling device. These results are predicated on establishing the best conditions for cell exposure as determined by flow cytometric analysis and a ^{51}Cr assay of vibriobactin treated-L1210 cells.

Materials and Methods

Vibriobactin was synthesized as previously described (9). A stock solution (2 mM) of vibriobactin was made in 50% (v/v) ethanol and water, and passed through a 0.2 μm filter prior to use.

Synthesis of (N-[3-(2,3-dimethoxybenzamido)propyl]-1,3-bis[2-(2,3-dimethoxyphenyl)-trans-5-methyl-2-oxazoline-4-carboxamido]propane), permethylated vibriobactin, was accomplished by the condensation of N^1,N^4-bis-[(2)-threonyl]-N^7-2,3-dimethoxybenzoyl) norspermidine with an excess of ethyl 2,3-dimethoxybenzimidate in refluxing methanol by methods similar to those worked out by Bergeron et al. (9) for the synthesis of vibriobactin.

Iron atomic absorption standard solution, 1003 μg/ml Fe in 2% HNO_3 (Aldrich, Milwaukee, WI) was used as an iron source.

Cell Culture: Murine L1210 leukemia cells and human Burkitt lymphoma cells (Daudi) were maintained in exponential growth as suspension cultures in complete medium containing RPMI-1640, HEPES-MOPS buffer, and 10% fetal bovine serum. Cultures in logarithmic growth were treated with vibriobactin and parabactin at concentrations ranging from 10^{-3} to 10^{-7} M. At intervals of 12 to 48 hr, cells were counted by electronic particle analysis (Coulter Counter, Model ZB1, Coulter Electronics, Hialeah, FL). The 50% inhibitory concentration (IC_{50}) was determined as the chelator concentration necessary to inhibit cell growth to 50% of the control growth at 48 hr. Viability was determined by trypan blue dye exclusion.

Preformed vibriobactin-iron chelates were formed by the addition of equimolar amounts of vibriobactin and iron to the media 30 min prior to the addition of the cells.

A 50% ethanol and water solution in appropriate amounts was used to treat cells in all control flasks. Addition of iron and ethanol alone in the concentrations used had no significant effect on growth studies.

Partition Studies: The partition equilibrium values for vibriobactin in octanol and phosphate buffered saline (PBS) were determined as previously (4).

Regrowth Studies: L1210 cells in suspension culture at approximately $3x10^5$ cells/mL were incubated in the presence of 10 μM vibriobactin for 5 hr at 37°C. The cells were then washed twice in fresh complete media and resuspended at a final concentration of $5x10^4$ cells/mL and incubated at 37°C. Cell samples during logrithmic growth were counted for up to 60 hrs after vibriobactin treatment and compared to controls.

Flow Cytometric Analysis: Cell analysis was performed with a FACS II flow cytometer (Becton Dickinson FACS systems, Sunnyvale, CA) interfaced with a microcomputer (Hewlett Packard 45B, Fort Collins, CO.). Cultured L1210 cells were treated with 10 μM vibriobactin, and incubated. Samples were removed at hourly intervals for 5 hrs and stained with propidium iodide (PI) (Calbiochem, San Diego, CA) and then exposed to RNase. DNA distributions were obtained from analysis of the red fluorescence from the PI-stained DNA (10).

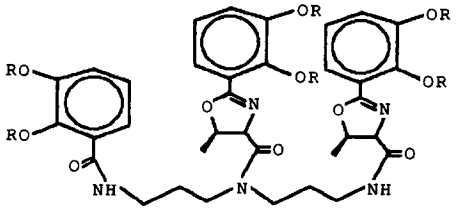
Bromodeoxyuridine (BrdUrd) Incorporation Studies: Cultured L1210 cells were treated with vibriobactin, washed, resuspended, and incubated as in the regrowth studies. At times, 0, 5, 10, 15 and 20 hrs of regrowth cell samples were removed for dual parameter flow cytometric measurments of cellular DNA content and amount of BrdUrd incorporated into cellular DNA by methods presented elsewhere (11,12).

Cr^{51} Release Assay: L1210 cells were treated with vibriobactin 10 μM for 5 hr, washed with fresh media and regrown for 20 hr. Approximately $2x10^6$ cells were centrifuged and 150 μCi Cr^{51} added to the pelleted cells. The pellet was incubated for 45 min. at 37°C in a 5% CO_2 atmosphere. Sample radiation was counted with an automatic gamma counter (LKB-Wallac RiaGamma 1274, Wallac Oy, Finland).

A microwell assay using triplicate samples of 200 μl containing $1x10^5$ cells was employed as described elsewhere (13). The % Cr^{51} release for the control cells was compared to that for the vibriobactin treated cells.

Results and Discussion

Vibriobactin, Figure 1, R=H, a hexacoordinate catecholamide iron chelator isolated from Vibrio cholerae by Neilands (8) and recently synthesized in our laboratories (9) is very active against L1210 cells in culture. The ligand is



R=H UIBRIOBACTIN
R=CH_3 PERMETHYLATED UIBRIOBACTIN

Figure 1

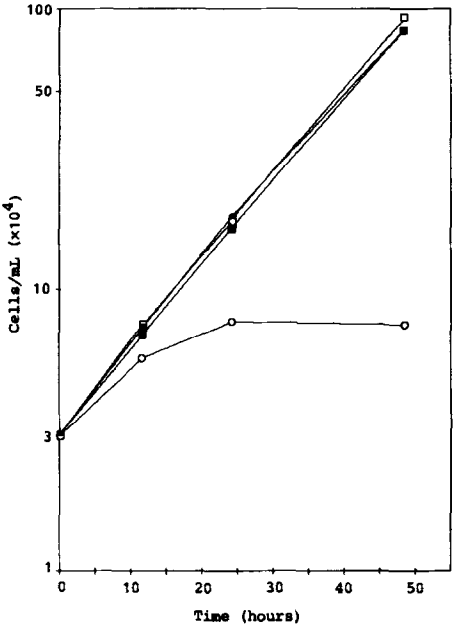


Figure 2. Growth properties of L1210 cells. Control (□), 10 μM vibriobactin (○), 10 μM, vibriobactin and 10 μM Fe^{3+} (■), 10 μM permethylated vibriobactin (●).

somewhat more lipid soluble than parabactin as indicated by its octanol-water partition constant of 65 vs. 35 for parabactin. It exerts pronounced effects on the growth characteristics of L1210 cells, Figure 2, displaying a 48 hr IC_{50} concentration of 2 μM. The effects of the ligand are clearly associated with its ability to chelate iron as when it is presented to the cells as the preformed iron chelate, even at 5 times the IC_{50}, its antimitotic activity is absent, Figure 2. Furthermore, that the compound's activity is associated with its ability to sequester iron is indicated by the fact that methylation of the ligands three catechol groups, Figure 1 R=-CH_3, eliminates its activity against L1210 cells, Figure 2.

The effects of vibriobactin on L1210 cells can be reversed by removing the chelator. When the cells are first treated with 10 μM vibriobactin for 5 hrs and then washed with fresh medium and resuspended they will grow although not nearly as fast as untreated cells. The washed cells display a 15 hr vs. 10 hr doubling time for up to 30 hrs after which cell growth parallels the control cell growth. The ligand however does demonstrate cidal activity on continued exposure of the cells

Table I
Cell Viability after Vibriobactin 10 μM Treatment

Time	Viability
zero (control)	>98%
10hr	96%
22hr	64%
30hr	61%
48hr	<10%

to 10 μM vibriobactin as indicated by trypan blue exclusion, Table 1. For example when cells are presented with vibriobactin for 24 hrs, approximately 35% of the cells die.

In an attempt to gain some insight into the mechanism of action of vibriobactin against L1210 cells the effects of the ligand on cell cycle kinetics were evaluated with flow cytometry. The cells were treated with 10 μM vibriobactin in complete media and aliquots removed every hour for DNA analysis for 5 hrs. There was a substantial effect on the cell cycle kinetics with a clear block at the G_1/S border after 3 hrs, Figure 3. The cell population in the G_1, S and G_2 + M phase is clearly altered over the 5 hr treatment, Figure 3.

The cells were next treated with 10 μM vibriobactin for 5 hrs and then washed with fresh media and resuspended. The change in growth characteristics of the cells were quite pronounced. If we take resuspension as time zero, after 5 hrs the

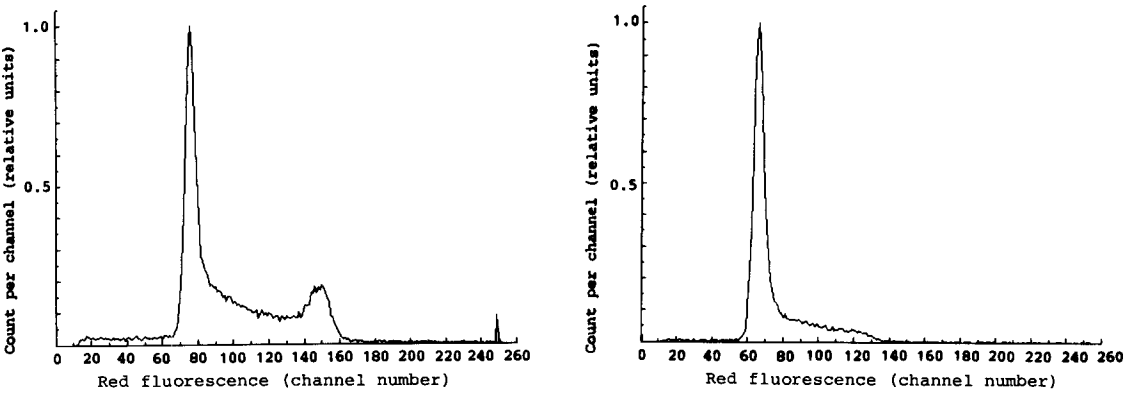


Figure 3. Flow cytometric DNA analysis of L1210 cells treated with 10 μM vibriobactin.

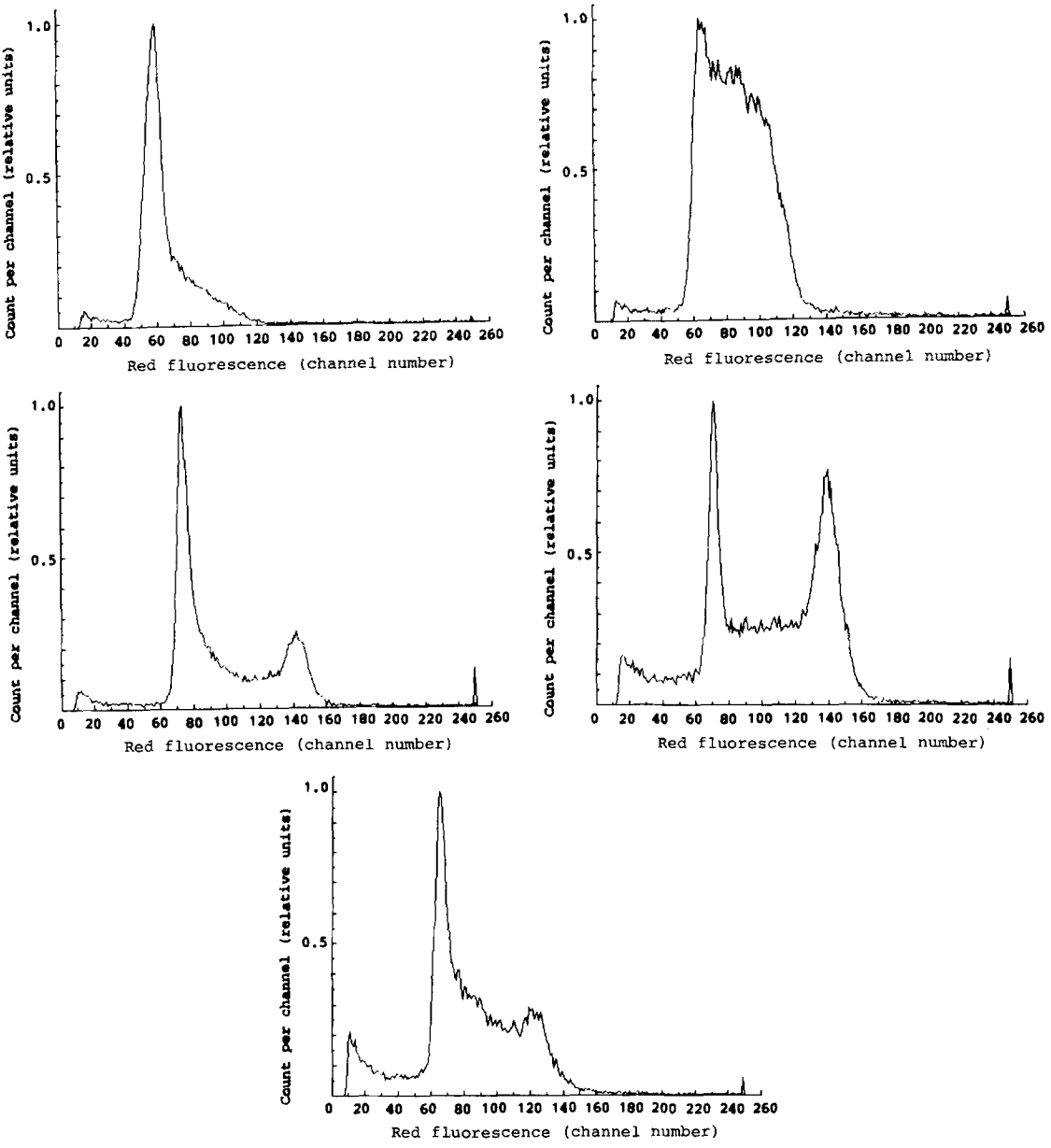


Figure 4. Flow cytometric DNA analysis of L1210 cells regrown in fresh media after treatment with 10 μM vibriobactin for 5 hrs.

change in phase ratios is enormous, Figure 4. It is clear from this data that the G_1/S block is released on removal of the drug. This progression through the phases continues for 20 hrs. Cell cycling was further substantiated by BrdUrd incorporation, Table II.

Table II

	% BrdUrd Incorporation	
Time	Control	Treated
0	44	16
5	50	47
10	53	45
15	47	16
20	49	32

To verify that the cell block was completely reversible and that decreased cell growth was not associated with chelator induced cell death a ^{51}Cr release assay was performed. The increase in ^{51}Cr released relative to controls was essentially nonexistant clearly establishing cell viability.

Finally, in an attempt to ascertain how broad an activity vibriobactin and parabactin have against tumor cells, we have chosen to test them against Daudi cells. As can be seen from Figure 5, both chelators have a substantial effect on the growth properties of the cells. Both ligands show 48 hr IC_{50} values of 2 μM.

In summary, the cells treated with vibriobactin are remarkably altered in their growth characteristics. The ligand has an impressive IC_{50}, 2 μM and under the con-

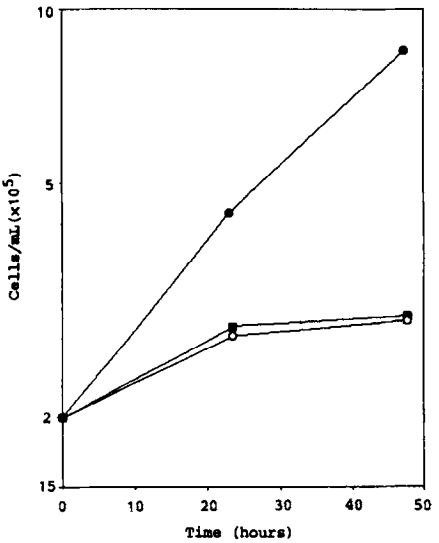


Figure 5. Growth properties of Daudi cells. Control (●), 5 μM parabactin (■), 5 μM vibriobactin (○).

ditions of this experiment is clearly a potent, reversible cell blocking and cell synchronization agent. Furthermore, when cells are exposed to the chelator for extended periods of time the ligand demonstrates considerable cidal activity.

References

1. Weinberg, E. D., Physiological Reviews 64:65 (1984).
2. Weinberg, E. D., Nutrition and Cancer 4:723 (1983).
3. Milder, M. S., Cook, J. D., Stray, S. and Finch, C. A. (1980) Idiopathic hemochromatosis, an interim report. Medicine (Baltimore) 59, 34-39.
4. Bergeron, R. J., Cavanaugh, P. F., Kline, S. J., Hughes, R. G., Elliott, G. T., and Porter, C. W., Biochem. Biophys. Res., Com., 121:848 (1984).
5. Cory, J. G. and Fleischer, A. E., Cancer Res. 39 4600 (1979).
6. Lederman, H. M., Cohen, A., Lee, J. W. W., Freedman, M. H. and Gelfand, E. W., Blood 64:748 (1984).
7. Cavanaugh, P. F., Porter, C. W., Tukalo, D., Frankfurt, O. S., Pavelic Zlatko and Bergeron, R. J., Cancer Res. 45 4754 (1985).
8. Neilands, J. B., Ony, S. A. and Peterson, T., J. Biol. Chem. 254 1860 (1979).
9. Bergeron, R. J., Garlich, J. R. and McManis, J. S., Tetrahedron, 41, 507 (1985).
10. Braylan, R. C., Benson, N. A. and Nourse, V. A., Cancer Res. 44, 5010 (1984).
11. Dolbeare, F., Gratzer, H., Pallavicini, M. G. and Gray, T. W., Proc., Natl. Acad. Sci. U.S.A. 80 5573 (1983).
12. Becton Dickinson Monoclonal Antibody Source Book, Section 3.80.1. Becton Dickinson Immunocytometry Systems, Mountain View, CA.
13. Grabstein, K., Chen Y. U., 1980. In vitro immune responses, p. 128-137. Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi (eds). W.H.Freeman and Company, San Francisco, CA.