MODE OF ACTION OF BREDININ WITH GUANYLIC ACID ON L5178Y MOUSE LEUKEMIA CELLS

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Moderate concentrations of bredinin (1.2×10^{-5} M) strongly inhibited growth of L5178Y cells, with the effect being reversed by guanylic acid (GMP). However, at higher concentrations of bredinin the inhibition was not reversed completely by GMP added in excess. Bredinin was cytocidal at concentrations above 2×10^{-5} M, but 5×10^{-5} M bredinin in the presence of excess GMP, bredinin was cytostatic.

Bredinin inhibited nucleic acid synthesis of L5178Y cells, but bredinin itself was not incorporated into the nucleic acid. Inhibition of nucleic acid synthesis was clearly reversed by GMP. Similarly chromosomal aberrations in L5178Y cells caused by bredinin were reversed by GMP. In contrast, the effect of a high concentration of bredinin on cell multiplication was not reversed by GMP. The modal volume of L5178Y cells increased during incubation in the presence of bredinin and GMP for 24 hours, 5×10^{-5} M bredinin with GMP causing a 70% increase in cell volume. This increase in cell volume was mainly due to an increase in the protein content of the cells.

The cytostatic effect of bredinin with GMP was reversed completely by adenosine-3′,5′-cyclic monophosphate (cyclic AMP). Other cyclic nucleotides and nucleotides were ineffective. The reversing effect of cyclic AMP on cell survival depended upon the concentration of GMP, and was not seen in the absence of GMP. It was concluded that cyclic AMP influences the secondary cytostatic effect of bredinin, and not the primary cytotoxic effect reversed by GMP.

As reported in previous papers[1,2,3], the new antibiotic bredinin (4-carbamoyl-1-β-D-ribofuranosyl imidazolium-5-olate) is a derivative of AICA-riboside. Bredinin strongly inhibits the growth of tumor cells in tissue culture, this inhibition being reversed by GMP. The cytolytic activity of bredinin is due to inhibition of the conversion of IMP to GMP in the purine nucleotide biosynthetic pathway.

GMP does not completely reverse the inhibitory activity of high concentrations of bredinin, an observation explained by bredinin acting at another site. This paper reports studies exploring the secondary effect of bredinin on L5178Y cells.

Materials and Methods

Bredinin was obtained as described previously[1]. Other materials were purchased as follows: adenosine-3′,5′-cyclic monophosphate from Sigma Chemical Company, St. Louis, U.S.A.; ^{3}H-thymidine and ^{3}H-uridine from New England Nuclear. ^{14}C-Bredinin (2.12 mCi/mM) was synthesized in our laboratory by the method described previously[4].

The mouse leukemia cell line, L5178Y, was grown at 37°C in FISCHER's medium supplemented with 10% dialyzed bovine serum, penicillin and streptomycin. In this medium, the L5178Y cells grew logarithmically, with a generation time of about 11 hours until they reached a cell density of 10^{6} cells/ml. FISCHER's medium and colcemid for chromosome study were purchased from Grand Island Biology Co., Ltd., U.S.A. Bovine serum was prepared in our laboratory. Routine tests for *Mycoplasma* in the serum gave negative results.

Cell numbers and cell volumes were determined in a Coulter model B electronic particle counter. The percentage of cultured cells that survived various treatments was determined by comparing cell numbers with those obtained under control conditions.

Growth rate was calculated using the following equations:

$$\text{Growth rate (\%)} = \frac{\log \frac{\text{cell count after incubation for 40 hours with test material}}{\text{cell count at the start of experiment}}}{\log \frac{\text{control cell count after incubation for 40 hours}}{\text{cell count at the start of experiment}}}$$

The syntheses of DNA and RNA in L5178Y cells were determined by measuring the incorporation of thymidine-^{3}H (1 μCi/ml) and uridine-^{3}H (1 μCi/ml), respectively into cold 20% TCA-insoluble precipitates. The precipitates were washed twice with 5% TCA and 1% sodium pyrophosphate, once with ethanol - ether (1: 1, v/v), once with ether, and then air dried. Radioactivity was then measured in a liquid scintillation counter after addition of 9 ml of toluene with POPOP (0.01 g/liter) and POP (6 g/liter).

For examination of chromosomal aberrations, cultures of L5178Y cells were incubated for 24 hours with the test compounds treated with 0.005 μg/ml of colcemid and fixed. Chromosomal preparations were obtained by the standard method, involving treatment with 1% sodium citrate, fixation in methanol - acetic acid (3: 1, v/v), drying in air, and GIEMSA staining.

The protein content of L5178Y cells was determined by the method of LOWRY *et al.*[5]. Variations in the amounts of protein per cell were estimated by the method of KURODA and FURUYAMA[6].

Results and Discussion

L5178Y cells were incubated for 40 hours in FISCHER's medium containing various concentrations of bredinin, with or without 2×10^{-4} M GMP. After incubation, cell numbers were determined. As shown in Fig. 1 growth of the cells was completely inhibited by 10^{-5} M bredinin, but with 10^{-5} M bredinin and 4×10^{-5} M GMP there was essentially no decrease in the viable count. However, at a higher concentration of bredinin (5×10^{-5} M), the growth inhibitory effect was not reversed by GMP, even when excess GMP was added.

As described previously[2], the effect of GMP in preventing growth inhibition increased up to a fixed concentration (4×10^{-5} M) of GMP with higher concentrations of GMP having no further protective effect, irrespective of how much bredinin was added to the culture medium. Therefore, the results indicate that at the higher concentrations, bredinin attacks some site other than that protected by GMP.

Fig. 2 plots the percent survival of L5178Y cells on incubation in normal FISCHER's medium after exposure to bredinin with or without GMP. Bredinin clearly exerts a cytotoxic effect on the cells in the absence of GMP. Cell survival on 40-hour incubation after 16-hour exposure to various concentration of bredinin decreased greatly with increasing concentrations of bredinin. No cells survived incubation with 2×10^{-5} M bredinin.

Fig 1. The interrelated effects of bredinin and GMP on growth of L5178Y cells.

Cells were incubated with various concentrations of bredinin with or without 4×10^{-5} M GMP for 40 hours. Cell numbers were counted with a Coulter counter.

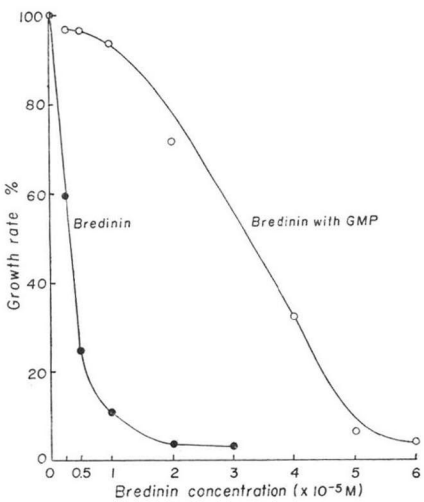


Table 1. Effects of bredinin with and without GMP on chromosomes of L5178Y cells

	Cells with abnormal chromosomes, %*	
	no GMP	4×10^{-5} M GMP
Control	0.91 (0)	1.94 (0)
Bredinin 10^{-5} M	86.29 (18.55)	0.89 (0)
Bredinin 10^{-4} M	99.12 (71.68)	1.49 (0)

* Percentages of cells in which all the chromosomes are fragmented are shown in parentheses.

In contrast, the survival of cells exposed to 5×10^{-5}M bredinin approached 100% in the presence of GMP (4×10^{-5} M). This observation suggests that bredinin has a cytostatic action on L5178Y cells when acting on a site not protected by GMP.

We reported previously[2] that 5×10^{-5} M bredinin strongly inhibited the synthesis of nucleic acids, and that the syntheses of DNA and RNA were inhibited to a similar extent. Inhibition of nucleic acid synthesis was reversed by GMP preserving cell viability but not reversing growth inhibition. Synthesis of macromolecule in the presence of growth inhibition was confirmed in experiments on chromosomal abnormalities.

The percentages of L5178Y cells with abnormal chromosomes after 24-hour exposure to either 10^{-5} M or 10^{-4} M bredinin are given in Table 1. GMP reduced the chromosomal aberrations that occurred in the presence of 10^{-4} M bredinin, a concentration that inhibits cell growth, but also at the higher bredinin concentration of 10^{-5} M. This observation indicates that synthesis of macromolecules continued for at least 24 hours even though cell numbers remained essentially constant. Considering that bredinin is a nucleoside antibiotic that acts primarily like an antimetabolite by blocking the conversion of IMP to GMP, the secondary site of action seems unique.

Fig. 2. Cytocidal and cytostatic effects of bredinin on L5178Y cells.

Cells were exposed to various concentrations of bredinin with or without 4×10^{-5} M GMP for 16 hours, washed with warm bredinin-free medium, and then incubated for 40 hours in bredinin-free normal medium.

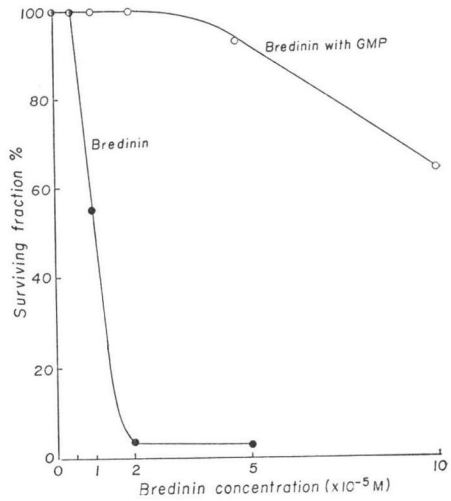
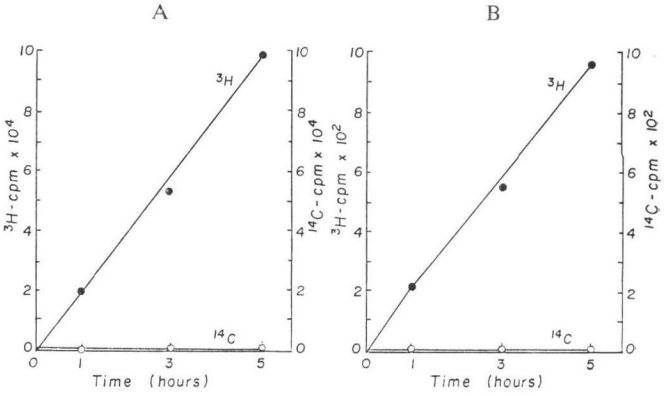


Fig. 3. Incorporation of labeled bredinin into nucleic acids of L5178Y cells.

Bredinin-^{14}C (2.12 mCi/mM) was added at time 0 at a concentration of 0.1 μCi/ml. Thymidine-^{3}H (1 μCi/ml, A) and uridine-^{3}H (1 μCi/ml, B) were added simultaneously.



^{14}C-Bredinin was added to exponentially growing cultures of L5178Y cells at a final concentration of 0.1 μCi/ml with the ^{3}H-labeled precursors thymidine and uridine. Fig. 3 shows that over a 5-hour period thymidine (Fig. 3A) and uridine (Fig. 3B) were incorporated linearly into the nucleic acids of L5178Y cells, while scarcely any labeled bredinin was incorporated. GMP was not present in the

medium.

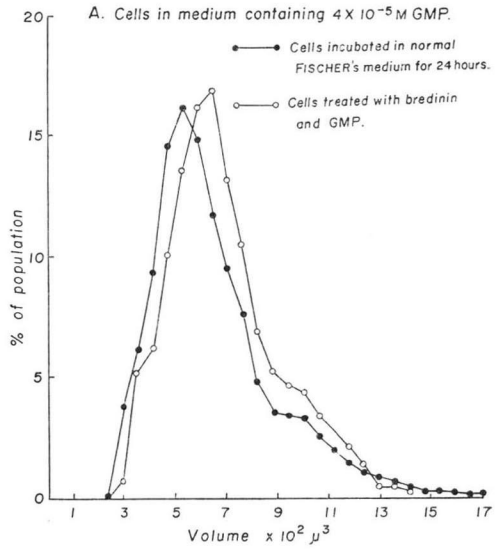
Furthermore we were unable to find bredinin-5′-phosphate or phosphoryl bredinin in the cells. These facts suggest that in the presence of GMP, nucleic acids are not damaged by lack of GMP as precursor or by insertion of bredinin into the molecules.

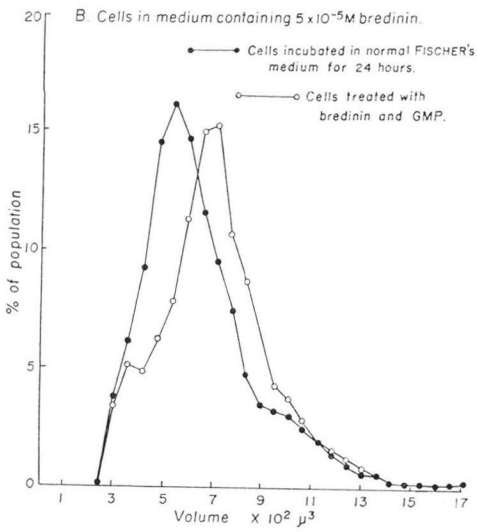
The continuing production of macromolecules in the absence of an increase in cell numbers when cells are incubated in the presence of 5×10^{-5} M bredinin and 4×10^{-5} M GMP predict that cell hypertrophy is occurring. Results of a study examining this premise are shown in Fig. 4. The size distribution of cells was measured in a Coulter Model B electronic particle counter with a reciprocal amplitude setting of 32, a reciprocal aperture current setting of 1, a tube of 100 μ diameter, and counting windows equal to 5 scale units. Size distributions of cells 24 hours after addition of 4×10^{-5} M GMP, of 5×10^{-5} M bredinin and of 5×10^{-5} M bredinin plus 4×10^{-5} M GMP are given in Figs. 4A, 4B and 4C respectively.

Addition of bredinin together with GMP resulted in about a 70% increase in volume. GMP alone caused little swelling, while bredinin alone caused rather less swelling than bredinin plus GMP. Furthermore, in the presence of both compounds, the cells did not die for at least 24 hours although cell growth was inhibited completely, as demonstrated in Fig. 2.

The effects of several antitumor agents[7,8,9,10] on cell size have been studied, and the rates of swelling reported were similar to the rate observed with bredinin plus GMP. It was at first thought possible that bredinin might cause meta-

Fig. 4. Effects of continuous exposure to bredinin and GMP upon the volume of L5178Y cells. Methods were as described in the text.





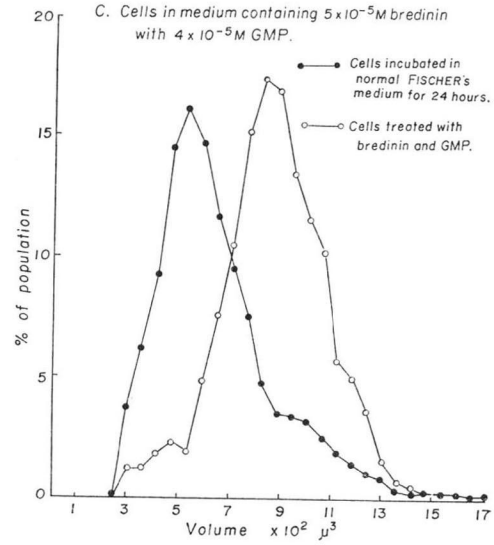
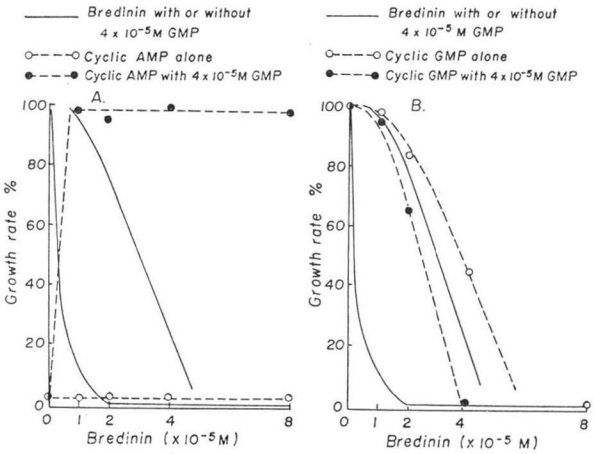


Table 2. Effects of bredinin plus GMP on the protein content of L5178Y cells

	$\times 10^{-4}$ μg protein/cell	
	no GMP	GMP(4×10^{-5} M)
Control	0.99 ± 0.039 (100.0%)	1.02 ± 0.012 (102.7%)
Bredinin (5×10^{-5} M)	1.23 ± 0.078 (124.5%)	1.46 ± 0.030 (147.5%)
Bredinin (10^{-4} M)	1.29 ± 0.025 (130.5%)	1.57 ± 0.107 (158.8%)

( ); % increase in size

Fig. 5. Effect of cyclic AMP and cyclic GMP on the inhibition of growth of L5178Y cells by varying concentrations of bredinin with or without GMP.
Various concentrations of bredinin, and 100 μg/ml of the cyclic nucleotide per ml with or without 4×10^{-5} M GMP were added to L5178Y cell cultures and the cells were incubated for 40 hours. Cell numbers were counted with a Coulter counter.
A; Effect of cyclic AMP. B; Effect of cyclic GMP.



phase arrest, with an increase of the mitotic index. However, our results did not agree with expected values for this. Therefore, it is concluded that bredinin does not inhibit the mitotic apparatus, like colchicine, or induce metaphase arrest. It is interesting that GMP alone also caused a slight swelling of the cells.

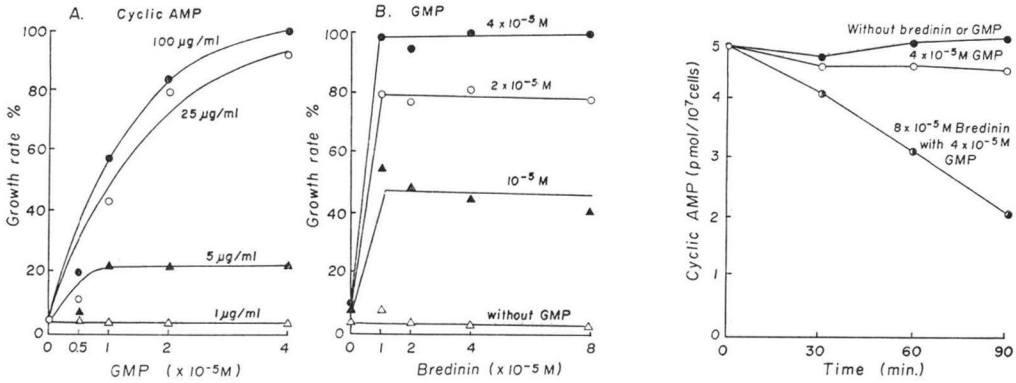
The reason for the increase in cell volume then was examined. The results obtained on the amount of protein in cells after treatment with bredinin with and without GMP (Table 2) suggest that hypertrophy of the cell volume is due in part to an increase in the amount of protein present. Probably an increase in the amounts of other macromolecules such as nucleic acid also occurs. These observations suggest that bredinin does not affect membrane transport of the cells since uptake of precursors required for synthesis of macromolecules is obviously occurring. In the presence of GMP alone, the amount of protein did not increase.

We investigated the distribution of L5178Y cells based on cell size for 48 hours after addition of 4×10^{-5} M GMP, 5×10^{-5} M bredinin or both, and also after exposure to these compounds for 24 hours and then incubation in normal FISCHER's medium for 24 hours. Although the presence of GMP for 48 hours did not result in swelling or lysis of the cells, the cells in medium containing bredinin lysed. When cells had been exposed to bredinin plus GMP for 24 hours and then incubated for 24 hours in normal FISCHER's medium, the size distribution of cells was identical with that of cells in control cultures. On the other hand, all cells incubated for 24 hours in FISCHER's medium after exposure to 2×10^{-5} M bredinin alone for 24 hours, also lysed, so that their size distribution could not be measured. These results indicate that in the presence of 10^{-4} M bredinin plus 4×10^{-5} M GMP most cells do not undergo irreversible damage within at least 24 hours. As mentioned above, several antitumor agents cause irreversible damage of cells, and result in moderate swelling like that seen with bredinin alone, but unlike the extensive hypertrophy observed with bredinin plus GMP. The large increase in cell size in the presence of 10^{-4} M bredinin and 4×10^{-5} M GMP must be the result of a continuation of the synthesis of macromolecules in the absence of cell growth.

Several studies[11,12,13] have established that cyclic AMP as well as compounds that raise the intracellular level of cyclic AMP can cause morphological enlargement of certain cell lines in tissue culture. These reports suggest that the changes in morphology induced by alteration of the cyclic AMP level

Fig. 6. Effect of GMP and cyclic AMP on the inhibition of growth of L5178Y cells by bredinin. L5178Y cells were incubated for 40 hours with bredinin (8×10^{-5} M) and various concentrations of GMP and cyclic AMP (A). The effects of various concentrations of bredinin and GMP with 100 μg/ml cyclic AMP were studied similarly (B).

Fig. 7. Effect of bredinin with GMP on cyclic AMP of L5178Y cells. Cells were incubated with 8×10^{-5} M bredinin and 4×10^{-5} M GMP for 30, 60 and 90 minutes.



could be due to inhibition of DNA synthesis. Although bredinin with GMP did not prevent DNA synthesis by the cells, the combination exerted a cytostatic effect similar to that of cyclic AMP.

Reversal of the effect of bredinin/GMP treatment was investigated with the results shown in Fig. 5. It was found that with the combination of 8×10^{-5} M bredinin, 4×10^{-5} M GMP, and 100 μg/ml cyclic AMP there was no decrease in the growth rate (Fig. 5A). However, in the absence of GMP, the effect of bredinin was not completely reversed by cyclic AMP because of cytotoxicity of cyclic AMP. Cyclic AMP is known to inhibit cell division[14] and, in the presence of GMP, cell survival was maintained by a cyclic AMP concentration at 100 μg/ml (Fig. 5A). Cyclic GMP also prevented the effect of bredinin, with its reversing potential approximately identical with that of GMP alone (Fig. 5B). Cyclic GMP was probably used by the cells as a substitute for GMP. The other cyclic nucleotides and nucleotides tested were ineffective.

The interrelationships of bredinin, GMP and cyclic AMP on cell growth are shown in Fig. 6. Cells were incubated for 40 hours in the presence of 8×10^{-5} M bredinin and various concentrations of GMP and cyclic AMP (Fig. 6A), and with 100 μg/ml cyclic AMP and various concentrations of bredinin and GMP (Fig. 6B). In the presence of 8×10^{-5} M bredinin, concentrations of both 25 μg/ml cyclic AMP and 4×10^{-5} M GMP were required to prevent growth inhibition, with lower concentrations of either GMP or cyclic AMP giving less protection (Fig. 6A). In contrast, the reversal of growth inhibition by the combination of GMP and cyclic AMP was not dependent on the concentration of bredinin (Fig. 6B). Therefore, it is concluded that cyclic AMP reverses the effect of bredinin as a function of GMP concentration because cyclic AMP prevents the action of bredinin on a site other than that protected by GMP. Possibly, bredinin together with GMP reduces the intracellular cyclic AMP level.

The hypothesis that bredinin plus GMP reduces the intracellular cyclic AMP level was confirmed by determining the concentration of cyclic AMP in cells using a cyclic AMP assay kit (The Radiochemical Centre, Amersham, England). A decrease in the cyclic AMP content of L5178Y cells on exposure to 8×10^{-5} M bredinin and 4×10^{-5} M GMP was detected after 30 minutes and continued linearly for the next 60 minutes (Fig. 7). GMP alone had little or no effect. These results support the

hypothesis that bredinin and GMP operate by reducing intracellular cyclic AMP since a constant level of cyclic AMP is required for cell division.

The primary mode of action of bredinin is blockage of the conversion of IMP to GMP[2]. The secondary effect appears to be concerned with interference with the intracellular cyclic AMP level. Cell enlargement must be due to inhibition of cell division only and not to inhibition of synthesis of DNA, RNA and protein. Although cyclic AMP has been reported to cause cell enlargement, the changes observed are similar to those caused by other compounds that inhibit cell division, and are not unique effects of cyclic AMP[11]. Therefore, it appears that the decrease of the intracellular cyclic AMP level results in inhibition of cell division, followed by cell enlargement.

Bredinin has a significant immunosuppressive effect, causing a decrease in the number of leucocytes[1]. However the decrease in the number of leucocytes is less than that caused by other immunosuppressive agents. The secondary site of action of bredinin observed in cell culture may be directly related to the suppression of the immunoresponse in rats. The serum concentration of bredinin after oral administration of an effective dose for immunosuppression is more than 8×10^{-5} M. Furthermore, GMP in the serum will partially reverse the primary site of action (Experiments on the reversal in tissue culture were carried out with dialyzed bovine serum).

If the immunoresponse is depressed by the cytostatic effect related to reduction of cellular cyclic AMP levels, it is easy to understand why the decrease in the number of leucocytes observed was less than that observed with other inhibitors of cell division. Further studies on the effects of bredinin will be reported in the near future.

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