EFFECT OF BREDININ AND ITS AGLYCONE ON L5178Y CELLS

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(Received for publication June 1975)

The aglycone of the nucleoside antibiotic, bredinin, was as strongly cytotoxic to L5178Y cells as bredinin. The cytotoxic properties of the aglycone were very similar to those of bredinin and the minimum inhibitory concentrations of both were 10^{-5} M. The growth inhibitory effects of both agents regardless of their concentrations, were reversed by guanylic acid, guanosine or guanine. However, on increasing the concentrations of these agents, the reversing effect of guanylic acid decreased gradually, the dose-response curves for the two agents being similar. Both agents inhibited the incorporation of thymidine and uridine, but not leucine into macromolecules in L5178Y cells and their inhibitory effects were reversed to similar extents by guanylic acid.

On the other hand, the growth inhibitory effect of the aglycone on L5178Y cells was prevented by adenine only, though not by adenosine or adenylic acid while the effect of bredinin was not prevented by adenine. These results suggest that the aglycone itself does not inhibit growth, but that its effect is due to its conversion to bredinin by an enzyme such as adenine phosphoribosyl transferase. For recovery of growth, three moles of adenine were required per mole of the aglycone. When the aglycone was administered orally to rats, bredinin was recovered in their serum and urine.

As described previously,[1,2] the new nucleoside antibiotic, bredinin (4-carbamoyl-1-β-D-ribofuranosyl imidazolium-5-olate), which is a derivative of AICAR-riboside, strongly inhibits the growth of tumor cells in tissue culture. Its growth inhibitory effect can be reversed by guanylic acid (GMP). It has been suggested that the cytolytic effect of bredinin may be due to its inhibition of the conversion of inosinic acid to guanylic acid in the purine nucleotide biosynthetic pathway, with resulting inhibition of the biosynthesis of macromolecules.

The present paper reports studies on the functional relationship between bredinin and its aglycone in L5178Y cells.

Materials and Methods

Bredinin was obtained as described previously[1] and its aglycone was synthesized chemically by the method of SCHIPPER and DAY.[3]

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 reach a cell density of 10^6 cells/ml. FISCHER's medium was 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 were counted in a Coulter counter (Coulter Electronics, Inc. Hialeah, Florida, U.S.A..) The percentage survival of cultured cells was determined as described in the previous paper.[2]

DNA, RNA and protein biosynthesis in L5178Y cells (2 ml) were estimated by measuring incorporation of thymidine-^{3}H (0.2 μCi/ml), uridine-^{3}H (0.2 μCi/ml), and leucine-^{3}H (4 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) and once with ether, and air dried. Then their radioactivity was measured in a liquid scintillation counter after addition of 9 ml of toluene containing POPOP (0.01 g/liter) and PPO (6 g/liter).

Results and Discussion

L5178Y cells in control cultures grew logarithmically to a density of approximately 10^6 cells/ml. Addition of bredinin or its aglycone markedly inhibited their growth. Fig. 1 shows the effects of increasing concentrations of bredinin and the aglycone upon the multiplication of L5178Y cells during 40 hours exposure to the drug in FISCHER's medium. The observed cell numbers are shown as percentages of the cell count (10^6 cells/ml) in control cultures after incubation for 40 hours. Growth was completely inhibited by 0.8×10^{-5} M concentrations of both agents. This indicates that both the aglycone and bredinin strongly inhibit growth of L5178Y cells.

Previously[2] it was found that above a relatively low concentrations of bredinin (10^{-5} M), a certain concentration of guanylic acid (GMP) could reverse the effect of bredinin, but that at higher concentrations of bredinin GMP could not reverse the effect of bredinin, completely. And when the concentration of bredinin was 5×15^{-5} M or more, even in the presence of GMP, bredinin caused loss of growth rate. However, Fig. 2 shows that the cytotoxicities of both the aglycone and bredinin were well reversed by GMP, and that both agents caused loss of growth rate. The dose-response curves for the two agents coincided well.

The effects of various nucleotides, nucleosides and bases on growth depression by these agents were next examined. The results showed that GMP at a concentration of 4×10^{-5} M considerably reduced the inhibitions of growth by both the aglycone and bredinin, and that guanosine and guanine also reduced their growth inhibitory effects. Fig. 3 shows the effects

Fig. 1. Effects of bredinin and its aglycone on growth of L5178Y cells.

Cells were incubated for 40 hours in FISCHER's medium containing various concentrations of bredinin or its aglycone, and then their growth was measured. Cell numbers were counted with a Coulter counter.

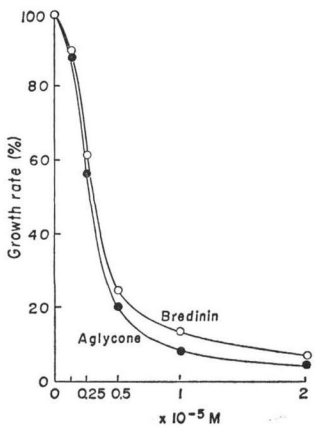
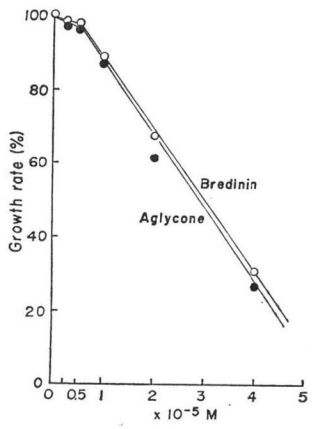


Fig. 2. Effect of GMP on the inhibitions of L5178Y cells by bredinin and the aglycone.

Various concentrations of bredinin and the aglycone were added to L5178Y cell cultures with 4×10^{-5} M of GMP and cell numbers were counted with a Coulter counter.



of incubating cells for 40 hours in medium containing various concentrations of the aglycone, with or without 5×10^{-5} M of adenine (A), adenosine (AR), or adenylic acid (AMP) on the cell numbers. Adenine reversed the growth inhibitory effects of the agents completely, even in the absence of GMP, while AR and AMP had very slight effects probably due to decreased phosphoribosyl transferase activity. Decrease of the activity may be accomplished by the conversion

Fig. 3. Effects of GMP, adenine (A), adenosine (AR) and AMP on inhibition of L5178Y cells by the aglycone.

Various concentrations of the aglycone with GMP 4×10^{-5} M, A 5×10^{-5} M, AR 5×10^{-5} M or AMP 5×10^{-5} M were incubated with L5178Y cells for 40 hours. The control contained only the aglycone. Cell numbers were counted in a Coulter counter.

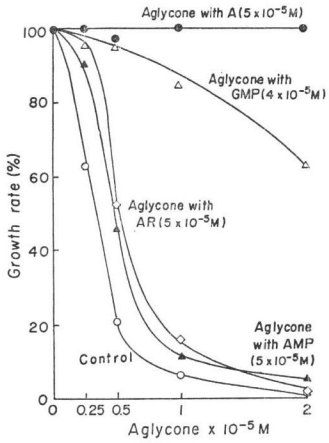


Fig. 4. Antagonism of adenine on the growth inhibitory effect of the aglycone on L5178Y cells.

L5178Y cells were incubated for 40 hours with the aglycone (10^{-5} M) and varioes concentrations of adenine. Cell numbers were counted with a Coulter counter.

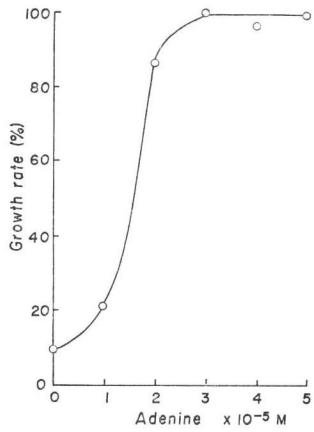


Fig. 5. Antagonistic effect of GMP on the growth inhibitory effects of bredinin and the aglycone on L5178Y cells.

L5178Y cells were incubated for 40 hours with bredinin or the aglycone and various concentrations of GMP. Cell numbers were counted in a Coulter counter.

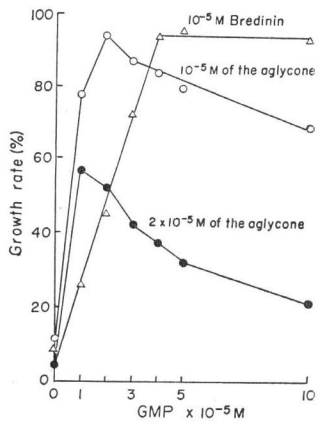
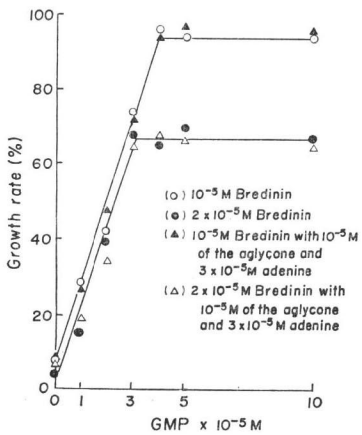


Fig. 6. Antagonistic effect of GMP on the growth inhibitory effect of bredinin with or without the aglycone and adenine on L5178Y cells.

L5178Y cells were incubated for 40 hours with various concentrations of GMP and bredinin with or without the aglycone and adenine. Cell numbers were counted in a Coulter counter.



of AR and AMP to hypoxanthine, with consequent competition of hypoxanthine and adenine for adenine phosphoribosyl transferase.

These results suggest that the aglycone itself has no growth inhibitory effect, and that its apparent effect results from its conversion to bredinin by an enzyme such as adenine phosphoribosyl transferase. Thus adenine antagonizes the effect of the aglycone.

Results on the antagonistic relation between the aglycone and adenine during cell reproduction are shown in Fig. 4. In this experiment cells were incubated for 40 hours with 10^{-5} M of the aglycone and various concentrations of adenine. To restore growth, three moles of adenine were required per mole of the aglycone.

Results on the antagonistic relation between the aglycone and GMP are shown in Fig. 5. The curve for the aglycone differed from that for bredinin, the minimum concentration of GMP requred for complete restoration of growth being considerably less with the aglycone than with bredinin. At higher concentrations of GMP the viable count decreased even when the concentration of the aglycone was kept constant at 10^{-5} M or 2×10^{-5} M. On the other hand, with both bredinin and the aglycone, a fixed concentration of GMP (2×10^{-5} M and 4×10^{-5} M, respectively) was required to prevent growth inhibition, and higher GMP concentrations had no further protective effect, irrespective of how much bredinin or the aglycone was added to the culture medium. With the aglycone, the effect of GMP decreased on increasing its concentration.

These results suggest that not all the aglycone is necessarily converted to bredinin and the difference in the effects of the two agents may depend on the rate of conversion of the aglycone to bredinin. However, evidence that much of the aglycone is converted to bredinin is reported later in this paper (Fig. 9). Further studies on this problem will be reported in the near future.

Fig. 6 shows the effect of GMP in preventing the cytotoxicity of bredinin in the presence of the aglycone. The conversion of the latter to bredinin was prevented by addition of adenine. The results coincide well with those on bredinin only. Namely, there is no evidence of a direct functional relationship between bredinin and the aglycone.

Next bredinin and the aglycone were added at a final concentration of 2×10^{-5} M to exponentially growing cultures of L5178Y cells at the same time as the radioactive precursors thymidine, uridine and leucine. Fig. 7 shows that bredinin and the aglycone strongly inhibited the synthesis of nucleic acid within 5 hours. The syntheses of DNA and RNA were inhibited

Fig. 7. Effects of bredinin and the aglycone on the synthesis of DNA, RNA and protein in L5178 Cells.

Bredinin and the aglycone were added at time 0 at a concentration of 2×10^{-5} M. Thymidine-^{3}H (0.2 μCi/ml, A and D), uridine-^{3}H (0.2 μCi/ml, B and E), and leucine-^{3}H (4 μCi/ml, C and F) were added simultaneously.

A, B and C; effect of bredinin.

D, E and F; effect of the aglycone.

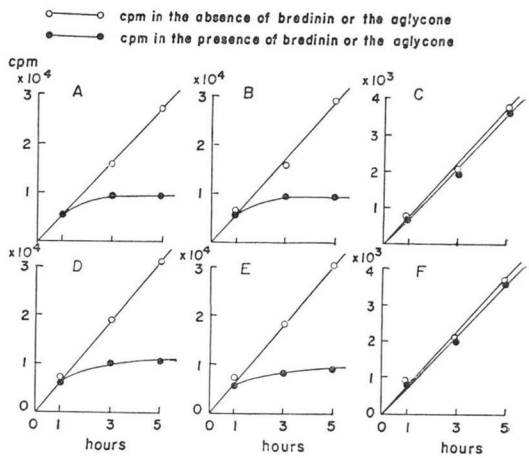


Fig. 8. Effects of bredinin and the aglycone with GMP on the synthesis of DNA, RNA and protein in L5178Y cells.

Conditions were as for Fig. 7, except that 4×10^{-5} M of GMP was added to the medium. Bredinin and the aglycone were added at a concentration of 6×10^{-5} M.

A, B and C; effect of bredinin.

D, E and F; effect of the aglycone.

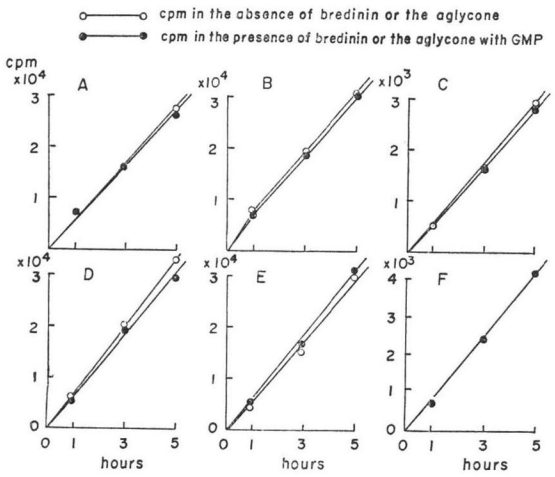
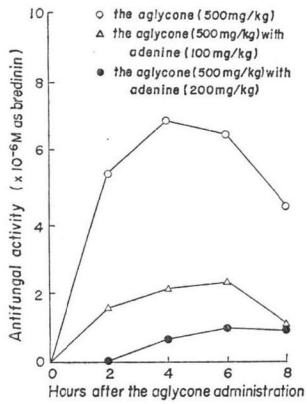


Fig. 9. Conversion of the aglycone to bredinin in rats, and effect of adenine on the coversion.

The aglycone (500 mg/kg) was orally administered to a rat (Wistar strain, ♂). Then serum specimens were collected at intervals, and the antifungal activity of the serum was determined. When added, adenine was administered simultaneously.



to similar extents. Moreover the inhibitory effects of both compounds had similar latent periods. Thus no difference was detected between the effects of bredinin and the aglycone.

Fig. 8 shows that the inhibition of nucleic acid synthesis by these agents was also reversed by GMP. Even with a concentration of bredinin or the aglycone of 6×10^{-5} M, at which GMP did not prevent reduction in the viability, GMP completely reversed the inhibition of nucleic acid synthesis by these agents. This particularly interesting finding was confirmed in other studies, such as experiments on chromosome abnormalities. These studies will be reported in the near future.

Direct evidence for conversion of the aglycone to bredinin was obtained as follows. Bredinin has strong antifungal activity (MIC, 4 μg/ml) on *Candida albicans*, while the aglycone has hardly any (MIC, 1,000 μg/ml or more). Therefore, if orally administered aglycone is converted to bredinin in rats, antifungal activity should be detected in the serum. Antifungal activity was determined by *in vitro* bioassay. As shown in Fig. 9 after oral administration of the aglycone antifungal activity increased gradually in the serum, reaching a maximum in 4 hours. On administration of adenine with the aglycone the antifungal activity was less than that on administration of the aglycone only (Fig. 9). We also obtained bredinin-like, crystalline material from the urine of the rats after oral administration of the aglycone. The purified material was confirmed to be bredinin from its mobility on thin-layer chromatography, and IR-, NMR- and UV-spectra. These results indicate that significant amounts of the aglycone are converted to bredinin in rats.

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