The Effects of Some Purine Analogs on the Growth of H.Ep. #1 and Chick Embryo Fibroblast Cells*

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SUMMARY

The substituted purine analogs 6-mercaptopurine, 6-thioguanine, 8-azaguanine, and the corresponding ribonucleosides 6-mercaptopurine riboside, 6-thioguanosine, and 8-azaguanosine are potent inhibitors of cell growth of H.Ep. #1 and chick embryo fibroblast cells in culture. H.Ep. #1 cells were approximately 100 times more resistant to 8-azaguanine than to 8-azaguanosine. In all other cases, for each cell line, sensitivity to the substituted purine base and its riboside was approximately the same. Chick embryo fibroblasts were 10-100 times more resistant to 6-mercaptopurine, 6-thioguanine, and their ribosides than were H.Ep. #1 cells. The inhibitory activity of all six compounds in both cell lines could be prevented by inosine or adenosine but not by guanosine or any of the pyrimidine metabolites tested. Clonal growth experiments confirmed results obtained with protein synthesis used as a parameter of cell growth. The data reported are in accord with the hypothesis that an active derivative of 6-mercaptopurine interferes with the conversion of inosinic to adenylic acid.

In the decade since their development the substituted purine analogs 6-mercaptopurine, 6-thioguanine, and 8-azaguanine have been the subject of considerable study. Their growth-inhibitory activity has been demonstrated in many microbial and neoplastic systems. These, as well as studies on the mode of action and mechanisms of resistance, have been extensively reviewed (9, 10).

As a continuation of our studies on growth inhibition of cells in culture by analogs of nucleic acid purines and pyrimidines, this report is concerned with the effects of 6-mercaptopurine, 6-thioguanine, 8-azaguanine, and the corresponding ribonucleosides on an epithelial line of human tumor origin.

To determine the degree of biological selectivity of an inhibitor in a host-virus system it is important to determine the effects of the compound on the host cell. Since chick embryo fibroblasts serve as host cells for a number of viruses, including the Rous sarcoma virus, parallel studies with these cells will also be included.

MATERIALS AND METHODS

Cultures of H.Ep. #1, a strain originally derived from a human cervical carcinoma and maintained continuously in culture (11), were grown in Eagle's medium supplemented with 10 per cent horse serum.

Second-passage chick embryo fibroblasts, prepared by a modification of the method of Dulbecco and Vogt (6), were grown in Eagle's medium containing double-strength amino acids and vitamins and supplemented with 5 or 10 per cent calf serum as suggested by Temin and Rubin (17). Tryptose phosphate, shown in preliminary experiments to reverse the inhibitory activity of some fluorinated pyrimidine analogs, was omitted from the medium.

Toxicity and reversal studies with both cell lines were carried out in Petri plate cultures following procedures previously described (13). Compounds were added after cell attachment, and incubation was continued for 7 days with renewal of media and compounds after 4 days. Growth

* This study was aided by research grants from the National Institutes of Health (C-8811) and the U.S. Atomic Energy Commission (AT (30-1)-910). It was presented in part at the 10th International Congress for Cell Biology, September, 1960, Paris, France.

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Received for publication June 7, 1961.
measured as total protein was determined by the method of Oyama and Eagle (12). Growth in control plates was taken as 100 per cent, with protein values at the time of addition of compounds representing zero growth. The experimental values shown in the charts represent the means of replicate cultures in at least two experiments.

For clonal growth studies $5 \times 10^4$ cells were plated on 60-mm. Petri plates engraved on the outer surface with a numbered grid. After incubation for 24 hours the position of approximately 30 cells was noted, and the appropriate compounds were added. At 24-hour intervals thereafter, the number of cells per colony for each of the 30 colonies was determined microscopically at $39 \times$.

The inhibitors employed were obtained from the Central Chemotherapy Office of this institute. Purity and concentration of stock solutions were checked by absorption measurements with a Beckman DU spectrophotometer.

RESULTS

Toxicity studies.—The effect of 8-azaguanine and its riboside 8-azaguanosine on chick embryo fibroblasts and H.Ep. #1 cells is shown in Chart 1. The per cent growth inhibition as compared with untreated control plates is plotted as a function of the inhibitor concentration. Toxicities of 8-azaguanosine for H.Ep. #1 cells and chick fibroblasts were almost identical. The similarity in response of these two cell lines did not hold for 8-azaguanine. H.Ep. #1 cells were approximately 100 times more resistant than chick embryo fibroblasts (Table 1).

With 6-mercaptopurine and 6-mercaptopurine riboside (Chart 2) chick embryo cells were approximately 100 times more resistant to both 6-mercaptopurine and 6-mercaptopurine riboside than were H.Ep. #1 cells (Table 1).

Results with 6-thioguanine and its riboside 6-thioguanosine (Chart 3) were similar to those for 6-mercaptopurine and its riboside in that the chick embryo fibroblasts were 100 times less sensitive than H.Ep. #1 cells to both 6-thioguanine and 6-thioguanosine. In each cell line 6-thioguanine and its riboside were equally effective (Table 1).

Effect of purine and pyrimidine metabolites.—The following experiments were carried out by exposing cells to a completely inhibitory concentration of antimetabolite plus selected concentrations of purine and pyrimidine metabolites.

The ability of the natural purine and pyrimidine derivatives to reverse or prevent inhibition may be summarized as follows: The pyrimidines thymine, cytosine, and uracil, as well as the corresponding ribonucleosides and deoxyribonucleosides, were completely ineffective in reversing inhibition. Reversal of all six inhibitors in both cell lines could be accomplished with inosine and adenosine at 37 $\mu$M, which was approximately 10 times the inhibitor concentration. This value may not represent the minimum concentration necessary for reversal. Reversal by guanosine (37 $\mu$M) was at best only very slight for any of the inhibitors tested.

To determine whether the interference with the toxicity of these inhibitors by adenosine or inosine was competitive or noncompetitive, a series of increasing concentrations of inosine or adenosine was tested against three concentrations of the inhibitor. The metabolite was always added at the same time as the inhibitor.

Results for the prevention of 6-thioguanosine inhibition by inosine in H.Ep. #1 cells may be seen in Chart 4A. Although the degree of preven-
tion appears to depend on both the concentration of the metabolite and the concentration of the inhibitor, a tenfold increase in 6-thioguanosine concentration required only a twofold increase in inosine to give comparable growth. Similar results were obtained for 6-thioguanosine vs. adenosine (Chart 4B) and 8-azaguanosine vs. adenosine with H.Ep. #1 cells (Chart 4C). These data suggest that the prevention of growth inhibition is somewhat competitive. A competitive relationship in the prevention of 6-mercaptopurine riboside inhibition by adenosine in chick embryo fibroblasts was more clearly defined. To achieve 50 per cent growth an increase in 6-mercaptopurine riboside concentration from 59 to 148 pM required an approximately twofold increase in adenosine concentration (Chart 4D).

**Time parameters**.—To determine the exposure time necessary for growth inhibition, cells were incubated in the presence of inhibitors for varying periods of time from 1/2 to 72 hours. After these time intervals the inhibitor was removed, and incubation was continued in fresh (inhibitor-free)
medium and also in fresh medium supplemented with inosine or adenosine. Incubation was continued for 5 days, at which time growth was compared with cells which had not been exposed to the inhibitor.

Results for 6-mercaptopurine, 6-thioguanine, 8-azaguanosine, and 6-thioguanosine with H.Ep. #1 cells and chick embryo fibroblasts may be seen in Chart 5. With all the compounds tested an exposure time of 25–50 hours was necessary for substantial inhibition. After 50 hours of exposure to 8-azaguanosine (3.2 μM), growth inhibition, as measured by the capacity to grow out in inhibitor-free medium, was not yet complete (Chart 5C). With 6-thioguanosine and appropriate concentrations used for each cell line (Chart 5B), the time parameters for growth inhibition of H.Ep. #1 and chick embryo fibroblasts were similar.

Clonal growth rate.—As indicated above, the addition of adenosine or inosine to an otherwise inhibitory concentration of the purine analogs resulted in growth (based on protein value) equal to that of controls. To determine whether the growth in the presence of the inhibitor plus metabolite was due to a normal growth rate by the whole population or to an increased growth rate by a fraction of the population, the growth (division) rates of individual members of the population were determined.

The results of an experiment (Chart 6) in which

![Chart 5](chart5.png)

**Chart 5.**—The effect of duration of exposure to 6-mercaptopurine, 8-azaguanosine, 6-thioguanine, and 6-thioguanosine on the subsequent growth of H.Ep. #1 and chick embryo fibroblast cells in inhibitor-free media in the presence and absence of inosine.
the multiplication rate for 30 individual cells was determined suggested that the former was correct. Despite the scatter it was obvious that the growth rates of all the colonies exposed to 6-thioguanosine plus inosine were relatively uniform and did not exceed the rate exhibited by the control. Scatter in subsequent experiments was no greater than that shown by the points plotted for 6-thioguanosine plus inosine.

It can be seen from the lowest curve representing the mean values for a similar number of single colonies that the concentration of 6-thioguanosine was sufficient for complete inhibition.

It is evident from Chart 7A that adenosine at 37 mM is growth-inhibitory under conditions of low cell density (5×10^4 cells/60 mm Petri plate). An equimolar concentration of inosine had no effect on cell division.

A comparison of the effects of the various purine analogs on the growth rate of H.Ep. #1 cells (Chart 7 B, C, and D) indicates a similarity between 8-azaguanine, 6-thioguanine, and 6-thioguanosine. Within 48 hours the number of cells per colony was equal to or less than the starting number. With 6-mercaptopurine and its riboside there was a two- and threefold increase by 72 hours, followed by a slow decline in the number of cells per colony until 168 and 192 hours (not shown), when the number of cells per colony was reduced to the number present before the addition of inhibitor.

In agreement with the protein data above, it is evident from Charts 6 and 7 that the presence of inosine prevented inhibition by all of the analogs tested.

DISCUSSION

Numerous studies on the mode of action of the purine antimetabolites 8-azaguanine, 6-mercaptopurine, 6-thioguanine, and their ribosides have revealed a variety of biochemical effects (9, 10). Unequivocal relationships between these effects and growth inhibition have yet to be demonstrated.

The similarity in inhibitory response to 6-mercaptopurine and its riboside, shown in Table 1, was similar to that shown for microbial and other mammalian cells in vitro and in vivo (8, 15, 18). Since there is evidence that with all three purine analogs the ribonucleotide is the active form (1, 2, 15), these results are in accord with the hypothesis that the 6-mercaptopurine riboside is either rapidly broken down to the purine base or that the ribonucleoside is converted to the ribonucleotide level at a rate similar to that of the base (9).

Szybalski and Smith reported a one-step mutation to hundred-fold resistance to 8-azaguanine in a strain of human bone marrow cells. This mutation had a high frequency and was reported to be relatively stable (16). H.Ep. #1 cells were approximately 100-fold more resistant to 8-azaguanine than to 8-azaguanosine. If this resistance to 8-azaguanine represents a mutation in our line of H.Ep. #1 cells, it is of interest to note that it was not accompanied by an increase in resistance to 6-thioguanine and 6-mercaptopurine relative to their respective ribosides.

In view of the suggestion that 6-mercaptopurine may exert its inhibitory activity after conversion to 6-thioguanine derivatives (9), it is interesting to note the similarity between 6-mercaptopurine and 6-thioguanine with respect to enhanced toxicity for H.Ep. #1 cells as compared with chick embryo fibroblasts (Table 1). With 8-azaguanine or its riboside this differential toxicity was not observed. Chick embryo fibroblasts were approximately 50 times more sensitive to 8-azaguanine than were H.Ep. #1 cells. Both cell lines were equally sensitive to 8-azaguanosine.

All the inhibitors tested exhibited a similar response to the presence of the purine and pyrimidine metabolites tested. The ability of inosine and adenosine and the inability of guanosine to prevent growth inhibition by all the antimetabolites in both cell lines was in agreement with the results.
The competitive reversal of 6-mercaptopurine inhibition by inosine in S-180, HeLa cells (8) and with *Bacillus cereus* (3) support the hypothesis that an active derivative of 6-mercaptopurine interferes with the conversion of inosinic acid to adenylic acid (5, 14). In accord with this hypothesis Hakala and Nichol further found that adenosine interfered with 6-mercaptopurine inhibition in a noncompetitive manner (8). However, with chick embryo fibroblasts a competitive interference was observed (Chart 4D). It was suggested from similar results with *Bacillus cereus* (3) that a competitive reversal by adenine was not in accord with the hypothesis that the primary site of inhibition was the pathway from inosinic acid to adenylic acid. However, in a complex system, although the primary site of inhibition were inosinic acid—adenylic acid, 6-mercaptopurine could at the same time competitively interfere with exogenous adenosine in the enzymatic reactions leading to the “active” ribonucleotide level. Thus, the resulting adenylic acid, responsible for the prevention of growth inhibition, would depend in a competitive manner on the relative concentrations of adenosine and 6-mercaptopurine, despite the fact that the above hypothesis for the primary

**Chart 7.—The effect of inosine, adenosine, 8-azaguanosine, 6-mercaptopurine, 6-mercaptopurine riboside, and 6-thioguanine, individually and in combination, on the growth of H.Ep. #1 cells.**
site of inhibition was correct. Likewise, the similarity in response to inosine and adenosine by all three inhibitors may reflect only their mutual requirement to be converted to the “active” ribonucleotide level, rather than identical sites of inhibition.

In earlier studies with the fluorinated pyrimidines (7), cells exposed to 5-fluorodeoxyuridine for as little as ½ hour would not grow out when transferred to fresh medium. Cells exposed to the inhibitor for as long as 20 hours would multiply if transferred to medium supplemented with thymidine, the reverser in this system. This phenomenon was subsequently shown to be related to unbalanced growth (4). As is evident from Chart 5 this phenomenon did not occur with the substituted purine analogs. The curves for cells exposed to the inhibitors for varying periods of time were the same whether or not cells were incubated in the presence of inosine after exposure to the inhibitor. Since the addition of inosine (or adenosine) had no effect when added after exposure to the inhibitor, it is suggested that inosine or adenosine prevent rather than reverse inhibition, as was the case with thymidine and the fluorinated pyrimidines.

Chick embryo fibroblasts exposed to 6-thioguanosine (16 μm) for 18 hours followed by incubation in fresh medium exhibited marked enlargement. Although there was no specific evidence of a lack of proportionality between protein and other parameters of cell growth in treated cells, the appearance of enlarged cells cautioned against the use of protein as the sole measurement of cellular growth. Consequently, a counting method for the determination of clonal growth rates with H.Ep. #1 was employed. As can be seen from Charts 6 and 7 a confirmation of inhibition and reversal data previously obtained by protein measurements resulted.

Adenosine, which prevented growth inhibition by the antimetabolites in experiments with approximately 3 x 10^5 cells per Petri plate, was inhibitory in clonal growth experiments in which a fivefold lower cell density was employed. The apparent contradiction is probably a reflection of protection arising from cell-to-cell interactions in denser populations. Similar observations with suboptimal growth media and other cellular inhibitors emphasize the importance of consideration of cell density in comparing results with other biological systems.

ACKNOWLEDGMENTS

The authors are indebted to Mrs. John Láñese and Miss Nancy Lederer for their assistance with clonal growth studies.

REFERENCES

The Effects of Some Purine Analogs on the Growth of H.Ep. #1 and Chick Embryo Fibroblast Cells

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