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SOME FACTORS AFFECTING THE TUMOR-INHIBITORY PROPERTIES OF COMBINATIONS OF AZASERINE AND 6-CHLOROPURINE*

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Abstract—The prolongation of the survival time of mice bearing sarcoma 180 ascites cells caused by combinations of 6-chloropurine and azaserine appeared to be insensitive to moderate increases in the dosage of azaserine. Maximum effectiveness of the combination of these agents occurred when either simultaneous administration of the two drugs was employed or the 6-chloropurine was given 1 hr after the azaserine; an interval of more than 3 hr caused a marked reduction in the degree of synergism. Therapy could be initiated up to 4 days after tumor implantation with some retention of antitumor properties by the combination. The ability of several purines and purine ribonucleosides to antagonize the chloropurine-induced enhancement of azaserine inhibition of sarcoma 180 was determined: guanosine prevented synergism at all levels of chloropurine tested, while adenosine, inosine, and hypoxanthine antagonized only at the lower levels of chloropurine administered. Adenine did not prevent drug enhancement. The results suggest that drug-induced blocks on the pathway to guanine nucleotides are associated with the synergism exhibited by combinations of 6-chloropurine and azaserine; however, an additional unknown site of blockade appears to be necessary for potentiation.

PREVIOUS findings have shown that 6-chloropurine has the capacity to enhance the chemotherapeutic effect of azaserine on a number of transplanted neoplasms.[1–3] It was presumed that the mode of action involved the simultaneous retardation of alternate pathways available for the biogenesis of purine nucleotides. Evidence is available which indicates that azaserine is an inhibitor of purine nucleotide synthesis *de novo* in malignant cells,[4–16] while measurement of the biochemical effects of chloropurine on purine metabolism indicates that the analog produces blocks both on the route to the formation *de novo* of nucleic acid guanine[15, 17] and in the area of purine catabolism, where xanthine oxidase and uricase have been shown to be sensitive to chloropurine and 6-chlorouric acid respectively.[18, 19] The probable site of the sensitive enzyme on the pathway to guanine nucleotides is the conversion of inosine 5′-phosphate to guanosine 5′-phosphate.[20] Contrary to the results obtained with other purine antagonists, the uptake of purines is not inhibited in tumor cells exposed to either chloropurine or a combination of chloropurine and azaserine.[17, 20, 21]

The drug-imposed blocks on these anabolic pathways presumably would lead to a marked decrease in the quantity of guanine nucleotides made available to the cell; the block by azaserine would prevent synthesis *de novo* and that by chlorpurine would retard the utilization of the adenine nucleotide pool. The utilization of any available

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preformed guanine as a compensatory response is probably minimal because of the presence of high levels of the catabolic enzyme, guanase, in the blood of mice.[22]

Nevertheless, attempts to correlate neoplastic sensitivity to combinations of chloropurine and azaserine with the ability of these agents to cause two distinct metabolic blocks on the guanine biosynthetic pathway suggested that these blocks may not in themselves be sufficient to account for sensitivity.[15] This report, therefore, describes studies of some of the factors affecting the synergic tumor-inhibitory properties of these agents that were initiated in an effort to gain information pertinent to an interpretation of existing biochemical data. Alterations in drug dosage, in sequence and in time of administration of drugs, and the effect of purines and purine ribonucleosides on drug synergy were investigated. The results are discussed in relation to possible mechanisms involved in the synergism seen with this combination.

MATERIALS AND METHODS

Experiments were performed on 9- to 11-week-old female Ha/ICR Swiss mice (A. R. Schmidt Co., Inc., Madison, Wis.). Transplantation of sarcoma 180 was carried out by withdrawing ascites fluid from a donor mouse bearing a 7-day tumor growth. The fluid was centrifuged for 2 min in a clinical centrifuge (1,600 $\times$ g), supernatant peritoneal fluid was decanted, a 10-fold dilution with isotonic saline was made, and 0·1 ml of the cell suspension (approximately 4 $\times$ 10^6 ascites cells) was inoculated intraperitoneally into each animal. Mice were distributed into groups of comparable weight and maintained during experiments on Rockland rat chow pellets and water *ad libitum*.

Chemicals were dissolved in isotonic saline, except for guanine and guanosine which were used as fine suspensions. Therapy was initiated 24 hr after tumor implantation in most cases, and treatments were then continued either once daily or twice daily, spaced approximately 12 hr apart, for 6 consecutive days. In each experiment tumor-bearing animals receiving injections of isotonic saline were included to serve as controls. Animals were weighed during treatment, and the weight change from the onset of therapy was used as an indication of drug toxicity.

Survival time was used as one of the criteria of tumor inhibition; mice surviving over 50 days and tumor-free animals were calculated as 50-day survivors in the determination of the average survival time. Complete regression of tumor growth was established by two criteria: drug-treated animals were maintained for over 100 days to allow any surviving ascites cells to become evident; ascites tumor-free animals were then autopsied for the presence of solid tumors in the peritoneal cavity. All gross nodules were examined histologically for the presence of malignant cells.

RESULTS

Previous studies have employed a fixed dose of azaserine, a quantity sufficient to produce essentially complete inhibition of the synthesis of purine nucleotides *de novo*, with varying amounts of chloropurine to estimate the chemotherapeutic efficacy of the combination in mice bearing ascites-cell neoplasms.[3] With sensitive tumors, the degree of inhibition by combinations of chloropurine and azaserine increased concurrently with increases in the dosage of chloropurine to a level at which drug toxicity presumably became a factor. It was of interest, therefore, to vary the quantity of azaserine with a fixed dose of chloropurine. The results of such an experiment are

presented in Table 1. As observed earlier,[3] chloropurine did not increase the survival time of mice bearing sarcoma 180 ascites cells, but azaserine caused a consistent lengthening of the life span of the tumor-bearing animals. Doses of azaserine from 0·2 to 4·0 mg/kg body weight produced similar prolongations of survival time when the azaserine was administered either by itself or with chloropurine.

TABLE 1. EFFECT OF COMBINATIONS OF 6-CHLOROPURINE AND AZASERINE ON THE SURVIVAL TIME OF MICE BEARING SARCOMA 180 ASCITES CELLS

Daily dose* (mg/kg)		Average survival (days)	No. of 50-day survivors†	No. of regressions†	Avg. Δ weight (g)‡
Azaserine	Chloropurine				
0	0	14·3	0/25	0/25	+6·0
0	20	11·0	0/10	0/10	+3·0
0	40	12·3	0/10	0/10	+3·4
0·2	0	17·8	0/10	0/10	+2·9
0·4	0	17·5	0/10	0/10	+2·5
2·0	0	18·6	0/10	0/10	+1·0
4·0	0	18·9	0/10	0/10	+1·5
0·2	20	22·1	1/15	1/15	+3·9
0·4	20	27·2	2/15	2/15	+5·3
2·0	20	24·2	0/14	0/14	+2·1
4·0	20	24·1	0/15	0/15	+0·9
0·2	40	31·8	4/20	4/20	+4·6
0·4	40	35·5	7/20	2/20	+4·4
2·0	40	34·2	3/15	2/15	−0·4
4·0	40	27·1	0/15	0/15	−0·9

* Administered once daily for 6 consecutive days, beginning 24 hr after tumor implantation, with combined treatments given simultaneously.

† Mice surviving over 50 days and tumor-free animals were calculated as 50-day survivors in determination of the average survival time (in all tables).

‡ Average weight change from onset to termination of drug treatment (in all tables).

The effects of sequence and time of administration of the two agents on the survival time of sarcoma 180 tumor-bearing mice were measured to determine the degree to which variations in drug administration would correlate with the duration of biochemical inhibition caused by each agent (Table 2). Simultaneous administration of the drugs or administration of chloropurine 1 to 3 hr after the azaserine dose caused a comparable lengthening of the survival time; however, separation of the two compounds by 6 hr appeared to have little advantage over azaserine alone. Alteration in the sequence of administration—that is, injection of chloropurine prior to azaserine—was much less effective.

Measurement of the effects of delayed therapy on the prolongation of survival time of sarcoma 180 tumor-bearing mice is shown in Table 3. In general, the greater the progression of tumor growth, the less effective was therapy, although treatments begun at 96 hr after tumor implantation, when mice were distended with ascites-cell growth, still resulted in a doubling of the survival time.

Table 4 shows the effect of some purine ribonucleosides on the synergism exhibited by combinations of azaserine and chloropurine. Azaserine, in combination with various concentrations of chloropurine, was employed at a dosage level producing essentially complete inhibition of the formation of purine nucleotides in sensitive ascites cells,[7, 11, 15] while the ribonucleosides were employed at a fixed level of 30

mg/kg. Neither the ribonucleosides nor chloropurine by themselves affected the survival time of tumor-bearing mice and these data are not included. Inosine prevented the chloropurine-induced enhancement of tumor inhibition by azaserine at the low dose of chloropurine; however, increasing the level of chloropurine resulted in restoration of synergism. Guanosine prevented the synergism exhibited by the drug

TABLE 2. EFFECT OF SEQUENCE AND TIME OF ADMINISTRATION OF 6-CHLOROPURINE AND AZASERINE ON THE SURVIVAL TIME OF MICE BEARING SARCOMA 180 ASCITES CELLS

Therapy*	Administration time* (hr)	Average survival (days)	No. of 50-day survivors	No. of regressions	Avg. Δ weight (g)
Controls		14·4	0/25	0/25	+6·5
Azaserine		19·5	0/20	0/20	−1·4
Azaserine + chloropurine	Simultaneous	34·2	7/25	6/25	+3·3
Azaserine + chloropurine	1	33·5	4/25	4/25	+3·5
Azaserine + chloropurine	3	29·1	3/25	3/25	+3·4
Azaserine + chloropurine	6	23·1	0/15	0/15	−2·8
Chloropurine + azaserine	1	24·1	0/15	0/15	+2·5
Chloropurine + azaserine	3	24·5	1/15	1/15	+3·0
Chloropurine + azaserine	6	18·8	0/10	0/10	+5·1

* Azaserine was administered at a level of 0·2 mg/kg and 6-chloropurine at a level of 40 mg/kg once daily for 6 consecutive days, beginning 24 hr after tumor implantation. When combination therapy was employed, the first listed drug was injected, and after the designated "administration time", the second drug was injected.

TABLE 3. EFFECT OF DELAYED THERAPY ON THE SURVIVAL TIME OF MICE BEARING SARCOMA 180 ASCITES CELLS

Therapy*	Time of initiation of therapy (hr)	Average survival (days)	No. of 50-day survivors	No. of regressions
Control		10·8	0/15	0/15
Azaserine + chloropurine	24	36·6	7/14	4/14
	48	36·7	7/15	3/15
	72	30·7	4/15	3/15
	96	20·9	2/15	1/15

* Azaserine was administered in a total daily dosage of 0·4 mg/kg simultaneously with 80 mg of chloropurine/kg injected as two equally divided doses given at intervals of 12 hr. Therapy was initiated 24, 48, 72, or 96 hr after tumor implantation and was continued for 6 consecutive days.

combination, and this effect appeared to be independent of the chloropurine dose. With doses of chloropurine up to 40 mg/kg, adenosine prevented the analog from enhancing the action of azaserine; at 60 mg chloropurine/kg, adenosine only partially prevented enhancement of tumor inhibition. Xanthosine did not decrease the synergism of any of the chloropurine–azaserine combinations.

The effect of some purines on the synergism exhibited by combinations of azaserine and chloropurine was measured, and the results are presented in Table 5. At the dose level employed (20 mg/kg), none of the purines prolonged the survival time of sarcoma-bearing mice. Hypoxanthine and guanine caused some prevention of synergy

TABLE 4. EFFECT OF PURINE RIBONUCLEOSIDES ON THE THERAPEUTIC SYNERGISM OF COMBINATIONS OF AZASERINE AND 6-CHLOROPURINE

Treatment*	Daily dose† (mg/kg)	Average survival (days)	No. of 50-day survivors	No. of regressions	Avg. Δ weight (g)
Control		12·7	0/75	0/75	+5·2
Aza	0·2	19·3	0/40	0/40	+2·1
Aza + ClP	0·2 + 20	27·5	1/15	1/15	+4·0
	0·2 + 40	28·5	9/69	8/69	+4·3
	0·2 + 60	30·9	7/30	7/30	+3·3
Aza + ClP + HxR	0·2 + 20 + 30	19·9	0/10	0/10	+2·8
	0·2 + 40 + 30	25·4	1/10	1/10	+4·4
	0·2 + 60 + 30	33·4	7/20	6/20	+4·2
Aza + ClP + GuR	0·2 + 20 + 30	20·8	0/10	0/10	+3·5
	0·2 + 40 + 30	23·6	0/10	0/10	+6·2
	0·2 + 60 + 30	21·8	0/10	0/10	+6·2
Aza + ClP + AdR	0·2 + 20 + 30	18·2	0/10	0/10	+3·9
	0·2 + 40 + 30	19·5	0/10	0/10	+3·6
	0·2 + 60 + 30	25·0	0/10	0/10	+4·3

* Abbreviations for Tables 4 and 5: Aza, azaserine; ClP, 6-chloropurine; HxR, inosine; GuR, guanosine; AdR, adenosine; Hx, hypoxanthine; Gu, guanine; and Ad, adenine.

† Administered once daily for 6 consecutive days, beginning 24 hr after tumor implantation, with combined treatments given simultaneously.

TABLE 5. EFFECT OF PURINES ON THE THERAPEUTIC SYNERGISM OF COMBINATIONS OF AZASERINE AND 6-CHLOROPURINE

Treatment*	Daily dose† (mg/kg)	Average survival (days)	No. of 50-day survivors	No. of regressions	Avg. Δ weight (g)
Control		12·0	0/35	0/35	+4·5
Aza	0·2	19·0	0/20	0/20	+2·0
Aza + ClP	0·2 + 20	26·4	1/20	1/20	+4·0
	0·2 + 40	27·5	3/25	3/25	+4·4
Aza + ClP + Hx	0·2 + 20 + 20	20·3	0/15	0/15	+3·4
	0·2 + 40 + 20	25·2	2/25	2/25	+3·2
Aza + ClP + Gu	0·2 + 20 + 20	22·9	0/15	0/15	+4·9
	0·2 + 40 + 20	27·1	2/15	1/15	+7·3
Aza + ClP + Ad	0·2 + 20 + 20	29·3	1/10	1/10	+4·7
	0·2 + 40 + 20	27·9	2/14	1/14	+5·7

* See note to Table 4.

† Administered once daily for 6 consecutive days, beginning 24 hr after tumor implantation, with combined treatments given simultaneously.

by azaserine and 20 mg chloropurine/kg. At the higher level of chloropurine, no significant decrease in synergistic tumor-inhibitory action was observed. Adenine, at the dosage level used, did not reduce the synergic effect of the drug combinations.

DISCUSSION

Measurement of the biochemical effects of 6-chloropurine on purine metabolism has indicated the presence of a drug-induced block on the pathway to guanine nucleotides.[15, 17, 20] This knowledge, coupled with the fact that inhibition of malignant cell growth by azaserine is compatible with the ability of this agent to inhibit the conversion of formylglycinamide ribonucleotide to formylglycinamidine ribonucleotide,[4–16] suggested that the synergy exhibited by combinations of chloropurine and azaserine might be attributable to the formation of two distinct metabolic blocks that limit the synthesis of guanine nucleotides by two alternative routes. In support of this hypothesis, the degree of growth inhibition of sarcoma 180 ascites cells by combinations of chloropurine and azaserine increased concurrently with increases in the dosage of chloropurine.[3] This correlated with a chemical measurement of the effect of the purine analog on the formation of polynucleotide guanine, for as the dose of chloropurine was increased, a concomitant increase in the percentage inhibition of guanine formation was observed.[17] This hypothesis was further tested by determining whether sensitivity to the drug combination correlated with the inhibition of guanine formation by each of these agents.[15, 21] Since a complete correlation was not obtained, other factors appear to be involved in the mode of drug action; the data, however, did not eliminate the necessity of blocks on the synthetic pathway to guanine-containing compounds for enhanced tumor-inhibitory properties.

In the ascites cell neoplasms used in these experiments, azaserine at a level of 0·2 mg/kg caused an essentially complete and irreversible inhibition of the purine biosynthetic pathway *de novo* for at least 12 hr.[8, 11, 15] Therefore, increasing the level of azaserine could not increase the degree of inhibition of this pathway. The observation that increasing the dose of azaserine also did not increase the effectiveness of azaserine–chloropurine combinations (Table 1) suggested that either the blockage of purine biosynthesis *de novo* by azaserine is associated with drug synergism or that a second important reaction inhibited by azaserine is also insensitive to increased levels of this agent. Since chloropurine cannot be injected 6 hr after the administration of the azaserine without a loss in the effectiveness of the drug combination (Table 2) and at this time purine biosynthesis *de novo* is completely inhibited by azaserine,[15] the metabolic blockade of purine biosynthesis *de novo* by azaserine cannot by itself be responsible for drug synergism. Either the two drugs simultaneously inhibit an unknown enzymatic reaction, or azaserine-induced inhibition of a metabolic step other than the conversion of formylglycinamide ribonucleotide to formylglycinamidine ribonucleotide is necessary for drug synergism.

It is reasonable to assume that the decreased synergy obtained when azaserine was administered after chloropurine was the result of a reversal of the chloropurine-induced inhibition of guanine nucleotide formation by products of the *de novo* biosynthetic route. The finding that 5-amino-4-imidazolecarboxamide can decrease the biological and biochemical efficacy of 6-chloropurine would support such a conclusion.[23]

The synergism observed with some combinations of azaserine and chloropurine was decreased or prevented by inosine, guanosine, or adenosine, while xanthosine did not antagonize the tumor-inhibitory effect of the drug combination (Table 4). The results obtained with inosine and adenosine suggest that a competitive relationship exists between these compounds and chloropurine, while the reversal exerted by guanosine at the dose level employed was independent of the chloropurine dose. These findings are in accord with the concept that a chloropurine-induced block between inosine 5′-phosphate and guanosine 5′-phosphate is required for the tumor-inhibitory properties of combinations of chloropurine and azaserine. The failure of xanthosine to prevent synergy does not fix the site of chloropurine-induced inhibition, since this molecule is rapidly catabolized and probably does not become phosphorylated to the nucleotide level. Hypoxanthine causes a reversal of the observed synergism at the lower dose of chloropurine; however, little could be concluded from the results obtained with guanine since considerable variation between individual experiments occurred. This presumably was the result of extensive catabolism by the guanases found in some mouse tissues.[22] Adenine does not affect the tumor-inhibitory properties of the drug combination even though the conversion of ^{14}C-labeled adenine to polynucleotide guanine is not retarded by the drug combination.[20, 21] The precise interpretation of this finding must await further investigation.

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