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COMPARISON OF SOME BIOLOGICAL AND BIOCHEMICAL PROPERTIES OF 6-BROMOPURINE AND 6-IODOPURINE

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Abstract—6-Bromopurine and 6-iodopurine each enhanced the carcinostatic activity of azaserine in a test system employing ascites cell forms of sarcoma 180 and Ehrlich carcinoma *in vivo*, a property previously demonstrated for 6-chloropurine. Hepatoma 134, Mecca lymphosarcoma, and a strain of sarcoma 180 resistant to combination therapy with azaserine plus chloropurine were relatively resistant to combination therapy with bromopurine and azaserine. Chloropurine, bromopurine, and iodopurine inhibited the incorporation of glycine-2-^{14}C into polynucleotide guanine of sarcoma 180 ascites cells; comparison of the magnitude and duration of inhibition of guanine nucleotide biosynthesis by these drugs indicated that on a molar basis chloropurine caused the strongest and iodopurine caused the weakest inhibition. These findings correlate with the relative ability of these compounds to synergize with azaserine in the inhibition of tumor growth. Results obtained in studies of the incorporation of hypoxanthine-8-^{14}C and guanine-8-^{14}C into polynucleotide guanine indicated in addition that the site at which the inhibition by bromopurine is induced resides between inosine 5′-phosphate and guanosine 5′-phosphate. These findings suggest that the 6-halogenated purines tested in combination with azaserine retard neoplastic growth by a similar mechanism, and that this appears to be related to retardation of the biosynthesis of guanine nucleotides.

6-CHLOROPURINE is one of the purine analogs having the capacity to inhibit growth of both a number of transplantable rodent neoplasms[1–3] and human leukemia.[4, 5] Furthermore, this agent markedly enhances the carcinostatic properties of the glutamine antagonist, azaserine.[6–8] Investigations designed to determine the biochemical lesions responsible for the synergic effects obtained with this drug combination have suggested that neoplastic sensitivity is associated, in part, with two metabolic alterations: a chloropurine-induced block that appears to reside between inosine 5′-phosphate and guanosine 5′-phosphate, and an azaserine-sensitive reaction on the biosynthetic pathway of purine nucleotides *de novo*.[9–11] These sites of inhibition may function to retard simultaneously the formation of guanine nucleotides via the *de novo* pathway and from the relatively large pool of adenine-containing nucleotides.

The pronounced carcinolytic activity of 6-chloropurine, when used in combination with azaserine, prompted the present study of some of the biological and biochemical properties of 6-bromopurine and 6-iodopurine in various ascites cell neoplasms, in order to determine whether these agents possess any significant advantages over 6-chloropurine as inhibitors of tumor growth.

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MATERIALS AND METHODS

Experiments were performed on 9- to 11-week-old female Ha/ICR Swiss mice (A. R. Schmidt Co., Madison, Wis.), AKD2F1 mice (Roscoe B. Jackson Memorial Lab., Bar Harbor, Maine), and C3H mice.* Transplantation of ascitic neoplasms was carried out by withdrawing peritoneal fluid from a donor mouse bearing a 7-day tumor growth. The suspension was centrifuged for 2 min (1600 × g), the supernatant peritoneal fluid was decanted, a 10× dilution with isotonic saline was made, and 0·1 ml of the cell suspension was injected intraperitoneally into each animal. In all experiments mice were maintained on Rockland rat chow pellets and water *ad libitum*.

Studies designed to assess the sensitivity of neoplastic cells to the agents employed were executed by determining the relative abilities of various types of therapy with the agents, both to prolong survival time and to cause regression of tumor growth; techniques and criteria previously described[12] were employed.

Measurement of drug-induced inhibition of various enzymic sequences involved in biosynthesis of purine nucleotides was carried out by treating each animal with a single intraperitoneal injection of either 6-chloropurine,† 6-bromopurine, or 6-iodopurine dissolved in isotonic saline; at selected times after this dose each mouse was injected intraperitoneally with either 100 μg of glycine-2-^{14}C (Tracerlab, Inc., Waltham, Mass.; 10^4 counts/min per μg), 50 μg of hypoxanthine-8-^{14}C (Nuclear-Chicago Corp., Chicago, Ill.; $9{\cdot}4 \times 10^3$ counts/min per μg), 50 μg of guanine-8-^{14}C (10^4 counts/min per μg), or 50 μg of adenine-8-^{14}C ($1{\cdot}7 \times 10^4$ counts/min per μg). (The last two compounds were obtained from the California Corp. for Biochemical Research, Los Angeles, Calif.) Guanine was allowed 20 min for incorporation; an interval of 1 hr was provided for the other isotopes. The cells were then harvested, and the nucleic acid purines and acid-soluble adenine were isolated and analyzed by the method of LePage.[13] Radioactivity was measured with a Nuclear-Chicago model D47 gas-flow counter equipped with a "micromil" window.

RESULTS

The effects of 6-bromopurine and azaserine on the survival time of mice bearing sarcoma 180 ascites cells were determined according to the method of an earlier study with 6-chloropurine and azaserine;[8] the results obtained are presented in Table 1. Although treatment with azaserine produced only a moderate prolongation of survival time and bromopurine *per se* had no effect on life span, combinations of bromopurine with azaserine caused significant extension of survival time of tumor-bearing mice, and the tumors in a number of the treated animals regressed. A comparison of these findings with those obtained earlier,[8] however, indicated that the bromopurine and azaserine regimen yielded fewer complete regressions than did therapy with optimal doses of chloropurine and azaserine.

Toxicity was observed in mice treated with combinations of 0·4 mg of azaserine/kg and 300 mg of bromopurine/kg, and this was evidenced by a pronounced weight

* C3H mice were bred in our laboratories (Noble Foundation). Initial breeding stock was donated by Dr. Howard B. Andervont and Dr. G. Burroughs Mider, National Institutes of Health.

† 6-Chloropurine was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio; 6-bromopurine was synthesized by the method of Beaman *et al.*,[18] and 6-iodopurine was synthesized by the method of Elion and Hitchings.[19]

loss and a survival time that was less than that produced by optimal doses of the agents in combination. In mice implanted with sarcoma 180 ascites cells, therapy with 0·4 mg of azaserine/kg and 160 to 200 mg of chloropurine/kg, administered daily as two equally divided doses for 6 consecutive days, resulted in an average survival time of 42·4 days and 60 per cent regression of tumor growth, although some toxicity,

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

Daily dosage* (mg/kg)		Average survival time (days)	No. of 50-day survivors†	No. of regressions†	Average △ weight‡ (g)
Azaserine	Bromopurine				
0	0	12·9	0/15	0/15	+7·0
0·4	0	22·1	0/15	0/15	+1·4
0	25	8·2	0/10	0/10	+6·9
0	50	10·2	0/10	0/10	+7·4
0	100	9·7	0/10	0/10	+4·6
0	200	8·8	0/15	0/15	+6·5
0	300	8·3	0/10	0/10	+7·0
0·4	25	38·5	3/10	2/10	+1·2
0·4	50	43·7	6/10	3/10	+1·4
0·4	100	44·8	7/10	4/10	+1·4
0·4	200	42·3	6/10	3/10	−0·8
0·4	250	32·2	2/5	2/5	+1·9
0·4	300	26·0	2/5	2/5	−2·6

* Therapy was initiated 24 hr after implantation of tumor cells, with the indicated daily dosage injected as two equally divided doses given at intervals of 12 hr for 6 consecutive days. Combination treatments were administered simultaneously.

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

‡ Average weight change from onset to termination of therapy.

TABLE 2. EFFECT OF DELAYED THERAPY ON THE SURVIVAL TIME OF MICE BEARING SARCOMA 180 ASCITES CELLS

Therapy*	Time of initiation of therapy (hr)	Average survival time (days)	No. of 50-day survivors†	No. of regressions†
Control		12·9	0/15	0/15
Azaserine + bromopurine	24	45·2	9/15	4/15
	48	40·3	5/10	2/10
	72	36·9	5/10	4/10
	96	16·2	1/10	0/10

* Azaserine was administered in a total daily dosage of 0·4 mg/kg simultaneously with 100 mg of bromopurine/kg, injected as two equally divided doses given at intervals of 12 hr. Therapy was initiated 24, 48, 72, and 96 hr after tumor implantation and was continued for 6 consecutive days.

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

which was characterized by a decrease in body weight of 10 per cent, was observed at the highest drug concentrations.

Table 2 shows the effects of delayed treatment on the chemotherapeutic effectiveness of a combination of bromopurine and azaserine, sarcoma 180 employed as the test system. In general, the longer therapy was delayed, the less pronounced was the response; however, significant prolongation of survival time was obtained when

treatments were initiated 72 hr after tumor implantation. Similar results have been obtained with combinations of chloropurine and azaserine.[14]

The growth-inhibitory properties of a combination of bromopurine and azaserine were measured in several ascitic neoplasms (Table 3); doses of compounds that

TABLE 3. EFFECT OF COMBINATIONS OF 6-BROMOPURINE AND AZASERINE ON THE SURVIVAL TIME OF MICE BEARING ASCITES TUMORS*

Neoplasm	Daily dosage (mg/kg)		Average survival time (days)	No. of 50-day survivors	No. of regressions	Average △ weight (g)
	Azaserine	Bromo-purine				
Ehrlich carcinoma	0	0	7·1	0/10	0/10	+5·4
	0·4	0	20·0	0/10	0/10	−1·2
	0	100	5·1	0/10	0/10	0·0
	0·4	100	41·1	5/10	3/10	+3·7
Sarcoma 180/Aza + ClP	0	0	14·5	0/10	0/10	+2·8
	0·4	0	24·0	0/10	0/10	+1·8
	0	100	9·3	0/10	0/10	+4·1
	0·4	100	23·4	0/10	0/10	+1·2
Mecca lymphosarcoma	0	0	9·7	0/10	0/10	+2·4
	0·4	0	11·8	0/10	0/10	+2·6
	0	100	10·5	0/10	0/10	+2·9
	0·4	100	13·1	0/10	0/10	+1·4
Hepatoma 134	0	0	17·8	0/10	0/10	−0·1
	0·4	0	23·9	0/10	0/10	−2·0
	0	100	13·0	0/10	0/10	−1·9
	0·4	100	19·6	0/10	0/10	−3·5

* Conditions were identical with those described in Table 1.

TABLE 4. EFFECT OF A COMBINATION OF 6-IODOPURINE AND AZASERINE ON THE SURVIVAL TIME OF MICE BEARING SARCOMA 180 ASCITES CELLS*

Daily dosage (mg/kg)		Average survival time (days)	No. of 50-day survivors	No. of regressions	Average △ weight (g)
Azaserine	Iodopurine				
0	0	12·8	0/10	0/10	+4·6
0·4	0	20·8	0/10	0/10	+1·0
0	117·6	10·5	0/10	0/10	+7·0
0·4	117·6	33·6	2/10	1/10	+2·0

* Conditions were identical with those described in Table 1.

yielded optimal results in sarcoma 180 were used. Synergism was observed when bromopurine and azaserine were injected into mice bearing the Ehrlich carcinoma, whereas the responses to the combinations were no better than those obtained with azaserine alone with sarcoma 180/Aza + ClP (a subline of sarcoma 180 that did not exhibit a synergistic response to combinations of azaserine and purine analogs), Mecca lymphosarcoma, and hepatoma 134.

Table 4 presents data from an experiment in which the effect of a combination of 6-iodopurine and azaserine on the survival time of mice bearing sarcoma 180 was measured. Treatment with iodopurine, 117·6 mg/kg, did not increase the survival

time of mice bearing this neoplasm, although a combination of this dose with azaserine, 0·4 mg/kg, resulted in an enhancement of the action of the glutamine analog.

A biochemical expression of the relative activities of the three halogenated purines studied is presented in Table 5. We measured the degree to which incorporation of glycine-2-^{14}C into mixed nucleic acid guanine was inhibited after treatment with various doses of each halogenated compound. This was done in order to determine the approximate dose of each compound that was required to inhibit biosynthesis of guanine nucleotides *in vivo* by 50 per cent. Chloropurine was the most active, bromopurine was intermediate, and iodopurine was the least effective in inhibiting the formation of guanine *de novo*.

TABLE 5. APPROXIMATE DOSE OF 6-HALOGENATED PURINE REQUIRED FOR 50 PER CENT INHIBITION OF THE BIOSYNTHESIS OF GUANINE *de novo*

Inhibitor	Dose (mg/kg)
6-Chloropurine	30
6-Bromopurine	60
6-Iodopurine	85

Tumor-bearing mice were each given a single intraperitoneal injection of various amounts of each purine analog; 1 hr later, 100 μg of glycine-2-^{14}C (10^4 counts/min per μg) was injected into each mouse and incorporation was permitted for 1 hr. Polynucleotide guanine was isolated, its specific radioactivity was determined, and the approximate dose for 50% inhibition of glycine incorporation into this base was then estimated.

Reduction in the amount of guanine formed, as a result of a single dose of chloropurine, was characterized by a duration of inhibition of purine synthesis that was longer than that observed with most other purine analogs; probably this is attributable to greater retention of chloropurine by mammalian tissues.[15] These findings prompted a measurement of the duration of action of 6-bromopurine on the biosynthesis of guanine *de novo* (Table 6). A dose of 50 mg of bromopurine/kg produced a relatively short-lived inhibition that was not measurable when the isotopic glycine was injected 3 hr after administration of the analog. This contrasts with the action of a molar equivalent of chloropurine, with which some retardation of guanine formation was demonstrable when glycine-^{14}C was administered 6 hr after the injection of drug.[9] An increase in the dose of bromopurine to 100 mg/kg yielded results similar to those obtained with the lower dose of chloropurine. The rate of incorporation of glycine-2-^{14}C into nucleic acid and acid-soluble adenine was not decreased by either level of bromopurine.

The effect of 6-iodopurine on the biosynthesis of purine nucleotides *de novo* is shown in Table 6. Administration to tumor-bearing animals of 58·8 mg of iodopurine/kg resulted in a transient depression of guanine biosynthesis; however, a larger dose, 117·6 mg/kg, caused a more pronounced inhibition. Contrary to the results obtained with chloropurine and bromopurine, some inhibition of glycine-2-^{14}C incorporation into acid-soluble adenine seemed to occur when the isotope was injected 1 hr after the iodopurine.

Table 7 shows the effect of bromopurine on the anabolism of the "physiological" purines. The rate of incorporation of hypoxanthine into polynucleotide guanine was depressed by 100 mg of bromopurine/kg, whereas the conversion of hypoxanthine to

adenine nucleotides was relatively insensitive to the drug. The utilization of guanine-8-^{14}C for the formation of polynucleotide guanine and adenine appeared to be increased in drug-treated cells. The conversion of adenine to polynucleotide guanine was also increased as a result of treatment with bromopurine.

TABLE 6. INCORPORATION OF GLYCINE-2-^{14}C INTO PURINES OF 6-BROMOPURINE- AND 6-IODOPURINE-TREATED CELLS

Drug	Dose (mg/kg)	Time after dosage with drug (hr)	Counts/min per μmole $\times 10^{-2}$		
			NA guanine	NA adenine	AS adenine
6-Bromopurine	50	0	5·1 ± 0·4	4·5 ± 0·3	86·6 ± 3·2
		1	2·5 ± 0·2	5·1 ± 0·3	84·8 ± 5·6
		3	5·3 ± 0·7	4·9 ± 0·3	94·8 ± 6·1
		6	5·7 ± 0·6	5·1 ± 0·2	100·7 ± 5·8
	100	0	5·1 ± 0·4	4·5 ± 0·3	86·6 ± 3·2
		1	1·3 ± 0·1	4·2 ± 0·4	66·5 ± 4·1
		3	2·8 ± 0·6	5·6 ± 0·4	90·3 ± 8·5
		6	4·5 ± 0·6	7·2 ± 0·5	103·3 ± 12·0
6-Iodopurine	58·8	0	5·5 ± 0·4	5·2 ± 0·3	105·7 ± 5·6
		1	3·3 ± 0·5	5·0 ± 0·6	89·7 ± 4·9
		3	5·3 ± 1·3	5·7 ± 1·4	110·4 ± 26·6
		6	5·3 ± 0·9	5·2 ± 0·4	106·3 ± 12·6
	117·6	0	5·5 ± 0·4	5·2 ± 0·3	105·7 ± 5·6
		1	1·7 ± 0·2	4·1 ± 0·4	69·6 ± 5·0
		3	2·2 ± 0·3	5·7 ± 0·6	105·4 ± 13·9
		6	6·5 ± 0·8	6·2 ± 0·5	116·3 ± 5·8

Tumor-bearing mice were injected with a single dose of 6-bromopurine or 6-iodopurine at the designated time; 100 μg of glycine-2-^{14}C (10^4 counts/min per μg) was administered to each mouse and incorporation was permitted for 1 hr. The zero time point represents the results obtained with control mice that received an injection of isotonic saline 1 hr before the radioactive glycine. Each figure represents the average (± standard error) of the results obtained from 8 mice. NA = nucleic acid; AS = acid-soluble.

TABLE 7. INCORPORATION OF HYPOXANTHINE-8-^{14}C, GUANINE-8-^{14}C, AND ADENINE-8-^{14}C INTO PURINES OF 6-BROMOPURINE-TREATED CELLS

Isotope	Bromopurine dose (mg/kg)	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Hypoxanthine-8-^{14}C	0	18·6 ± 4·7	28·6 ± 5·4	413·8 ± 84·0
	50	22·6 ± 0·9	37·5 ± 3·2	534·5 ± 53·9
	100	10·2 ± 1·4	34·8 ± 1·5	587·3 ± 66·1
Guanine-8-^{14}C	0	29·8 ± 8·6	2·5 ± 0·7	85·0 ± 14·7
	50	33·5 ± 8·7	3·4 ± 0·7	115·3 ± 2·9
	100	66·0 ± 9·9	4·8 ± 0·8	127·7 ± 12·1
Adenine-8-^{14}C	0	5·8 ± 0·6	76·8 ± 4·7	890·4 ± 54·3
	50	11·9 ± 2·9	96·4 ± 9·2	1133 ± 105·4
	100	8·9 ± 1·5	77·1 ± 9·2	1061 ± 121·0

Tumor-bearing mice were injected with a single dose of 6-bromopurine; 1 hr later, either 50 μg of hypoxanthine-8-^{14}C ($9·4 \times 10^3$ counts/min per μg), 50 μg of guanine-8-^{14}C (10^4 counts/min per μg), or 50 μg of adenine-8-^{14}C ($1·7 \times 10^4$ counts/min per μg) was administered to each mouse. The zero time points represent the results obtained with control mice that received an injection of isotonic saline 1 hr before the radioactive substrate. Each figure represents the average (± standard error) of the results obtained from 4 to 8 mice. NA = nucleic acid; AS = acid-soluble.

DISCUSSION

The pronounced antitumor activity of combinations of chloropurine and azaserine indicated the need to measure the activities of other 6-halogenated purines in combination with azaserine, even though earlier studies had shown that 6-bromopurine and 6-iodopurine exert only minimal inhibitory action on the growth of subcutaneous implants of sarcoma 180.[16]

Therapy with combinations of bromopurine and azaserine exerted pronounced antineoplastic activity, but the number of regressions observed in sensitive neoplasms was less than that obtained with chloropurine and azaserine.[8] Furthermore, chloropurine enhanced the action of azaserine over a wider range of doses than was observed with bromopurine. Toxicity, as measured by pronounced weight loss, appeared to be similar for molar equivalent levels of dosage with bromopurine or chloropurine when each was employed with azaserine. A combination of iodopurine and azaserine yielded a synergistic response in sarcoma 180 tumor-bearing mice, although this response was inferior to that obtained with the other halogenated purines.

The inhibitory activity of the 6-halogenated purines in combination with azaserine exhibited identical specificity in a spectrum of transplanted neoplasms, a circumstance suggesting that the mechanism by which the 6-halogenated purines potentiate the antineoplastic activity of azaserine is similar. The enhancement of the carcinostatic action of azaserine by chloropurine appears to be associated in part with a drug-induced block on the biosynthetic pathway of guanine nucleotides.[10] The relative inhibitory potencies of the various halogenated purine–azaserine combinations on the growth of sarcoma 180 support the concept that inhibition of the formation of guanine is important for carcinolysis, since the intensity and duration of interference with the biosynthesis of guanine correlated with the ability of the purine analogs to synergize with azaserine. The differences in the inhibitory potencies of these compounds may be the result of differences in the affinity for the sensitive enzyme, in the rate of transport to the enzymic site, or in the rates of metabolic transformation.

The decrease in the rate of conversion of hypoxanthine-8-^{14}C to polynucleotide guanine, accompanied by a lack of inhibition of the incorporation of guanine-8-^{14}C into the polynucleotide fraction by bromopurine, suggests that the target enzyme resides between inosine 5′-phosphate and guanosine 5′-phosphate; a similar site of action was postulated for chloropurine.[11] Furthermore, the observed stimulation by bromopurine of the incorporation of radioactive adenine and guanine into polynucleotide guanine was similar to the compensatory response seen with chloropurine.[9, 17]

The 6-halogenated purines may be considered antimetabolites of hypoxanthine, and the antineoplastic activity of these analogs can be correlated with the electronegativity or electron-withdrawing effect of the halogen atom on the purine ring and with the relative size of the halogen atom. These properties would emphasize the importance of synthesizing and testing 6-fluoropurine, since the fluorine atom should more closely resemble the oxygen atom in electronegativity and size than do the other halogens.

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