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INHIBITION OF THE BIOSYNTHESIS OF PURINES *DE NOVO* BY 6-CHLOROPURINE AND AZASERINE IN SENSITIVE AND NATURALLY RESISTANT ASCITES CELLS*

A. C. SARTORELLI, J. R. AKERS and BARBARA A. BOOTH

Biomedical Division of The Samuel Roberts Noble Foundation, Inc., Ardmore, Oklahoma

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Abstract—The action of 6-chloropurine and azaserine on the biosynthesis of guanine *de novo* was measured in five ascites-cell neoplasms to determine whether sensitivity to combinations of these agents could be correlated with the blockage of this pathway by the drugs. A positive correlation was obtained with four of these tumors. Azaserine caused essentially complete inhibition of purine biosynthesis, while chloropurine produced a maximum of about 70 per cent retardation of glycine incorporation into polynucleotide guanine in Ehrlich carcinoma and sarcoma 180, neoplasms sensitive to therapy with combinations of chloropurine and azaserine. The 6C3HED and the Mecca lymphosarcomas were relatively resistant to therapy with the drug combination. In the 6C3HED lymphosarcoma, azaserine produced a marked inhibition of purine nucleotide formation. Chloropurine did not affect the incorporation of glycine into polynucleotide guanine; however, it did afford approximately 40 per cent reduction in the rate of isotope utilization for polynucleotide adenine in this tumor. In the Mecca lymphosarcoma, both drugs were unable to affect guanine formation *de novo* to the same extent found in the sensitive neoplasms. However, with hepatoma 134, both azaserine and chloropurine caused a reduction in the rate of guanine formation comparable to the inhibition obtained in sensitive tumors, although this neoplasm is not sensitive to the drug combination. The possible interpretations of these findings have been discussed.

INTRODUCTION

6-CHLOROPURINE has been found to potentiate the tumor inhibition produced by the glutamine analogs, azaserine (O-diazoacetyl-L-serine) or DON (6-diazo-5-oxo-L-norleucine), in a number of experimental tumor lines.[1, 2] Studies on the mode of action of azaserine in mammalian systems have indicated that this agent produced intensive inhibition of the biosynthesis of purines *de novo*.[3–10] Since earlier work[11, 12] showed that 6-chloropurine retarded the incorporation of radioactive glycine into polynucleotide guanine without altering the rate of incorporation of glycine into adenine nucleotides, the possibility therefore existed that the tumor-inhibitory properties of combinations of chloropurine and azaserine were due to the formation of two mutually dependent blocks which limited the synthesis of guanine nucleotides. Simultaneous

* The following abbreviations are used: IMP for inosine 5′-phosphate, NA for nucleic acid, and AS for acid-soluble.

administration of these two drugs was essential for carcinostasis.[2] This finding was consistent with the theory that these agents established complementary metabolic blocks on pathways leading to purine nucleotide biosynthesis. To determine whether these biochemical events were related to the tumor-inhibitory properties of the drug combination, the effects of azaserine and chloropurine on the formation of purines *de novo* were compared in susceptible and naturally resistant ascites tumors.

MATERIALS AND METHODS

Five mouse ascites-cell tumors were employed in these experiments: sarcoma 180 and Ehrlich carcinoma in female Ha/ICR Swiss mice (A. R. Schmidt Co., Inc., Madison, Wis.), 6C3HED lymphosarcoma and hepatoma 134 in female C3H mice (Texas Inbred Mice Co., Houston, Tex.), and Mecca lymphosarcoma in female AKR mice (Texas Inbred Mice Co.). Animals were inoculated intraperitoneally six

TABLE 1. THE BIOSYNTHESIS OF PURINES *de novo* IN SARCOMA 180 ASCITES CELLS TREATED WITH AZASERINE

Hours after azaserine dose	Counts/min per μmole $\times 10^{-2}$		
	NA guanine	NA adenine	AS adenine
0	4·0±0·4*	3·3 ±0·5	58·3±5·0
1	0·1±0·05	0·07±0·1	0·1±0·03
3	0·1±0·04	0·08±0·03	0·5±0·1
6	0·2±0·1	0·1 ±0·01	4·0±1·4
12	0·4±0·1	0·3 ±0·1	7·3±1·2
24	0·7±0·04	0·4 ±0·05	7·6±1·1

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with 0·2 mg azaserine per kg. At the designated time, 100 μg glycine-2-^{14}C (10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time point represents the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive glycine.

days before use with 0·1 ml of ascites cell suspension prepared by centrifuging the cells for 2 min in a clinical centrifuge (1600 *g*) and suspending the packed cells in 10 vols. of isotonic saline.

Drugs were dissolved in isotonic saline and injected intraperitoneally: azaserine at a dose of 0·2 mg/kg and chloropurine (Nutritional Biochemicals Corp., Cleveland, Ohio) at a level of 40 mg/kg. Control animals received a comparable volume of isotonic saline in place of the drug. At various times after this dose, each mouse received an intraperitoneal injection of 100 μg of glycine-2-^{14}C (Tracerlab, Inc., Waltham, Mass.) (10^4 counts/min per μg). One hour later, the cells were harvested, and the nucleic acid purines and acid-soluble adenine were isolated and analyzed as described by LePage.[13] Radioactivity was measured with a Nuclear-Chicago Model D47 windowless gas-flow counter. Each experimental point represents the average of values obtained from the separate analyses of ascites cells from eight to twelve mice.

RESULTS

The effect of azaserine on the biosynthesis of purines *de novo* in sarcoma 180, a neoplasm in which chloropurine enhances the carcinostatic effect of azaserine, is shown in Table 1. Glycine-2-^{14}C incorporation into polynucleotide purines was used as the measure of purine synthesis. Injection of radioactive glycine 1 hr after the administration of azaserine resulted in essentially complete inhibition of glycine incorporation into nucleic acid purines and acid-soluble adenine. More than 80 per cent inhibition of this pathway remained 24 hr after the azaserine dose. In this neoplasm, chloropurine administration 1 hr prior to glycine-2-^{14}C resulted in 75 per cent inhibition of isotope incorporation into nucleic acid guanine, with inhibition persisting up to 6 hr after the chloropurine dose.[12]

The effects of these two agents on purine formation were studied using the Ehrlich carcinoma, a tumor which resembled sarcoma 180 in its sensitivity to combinations of these drugs. The results obtained are presented in Table 2. The findings parallelled

TABLE 2. THE BIOSYNTHESIS OF PURINES *de novo* IN EHRLICH CARCINOMA ASCITES CELLS TREATED WITH AZASERINE OR CHLOROPURINE

Drug	Hours after drug dose	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Azaserine	0	7·3±1·1*	5·3 ±2·1	105·6±10·9
	1	0·2±0·1	0·04±0·01	0·2± 0·07
	12	1·5±0·1	1·0 ±0·2	26·6± 2·7
	24	1·6±0·3	0·9 ±0·2	21·0± 3·3
Chloropurine	0	7·9±1·2	5·2 ±0·5	113·8± 7·3
	5 (min)	3·3±0·3	6·5 ±0·8	125·5±16·5
	1	2·9±0·4	4·8 ±0·6	102·7±12·5
	6	6·0±0·6	6·4 ±0·5	126·4± 4·8

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with either 0·2 mg azaserine per kg or 40 mg chloropurine per kg. At the designated time, 100 μg glycine-2-^{14}C (10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time point represents the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive glycine.

those obtained with sarcoma 180; azaserine produced marked, prolonged inhibition of purine synthesis, and chloropurine produced a maximum retardation of glycine incorporation into nucleic acid guanine of 63 per cent. No inhibition of the formation of polynucleotide adenine occurred in chloropurine-treated cells.

Table 3 presents the results of the experiments using 6C3HED ascites cells. Chloropurine was unable to enhance the tumor-inhibitory properties of azaserine in this lymphosarcoma. Azaserine treatment produced essentially complete inhibition of glycine incorporation into cellular purines, with marked inhibition still present up to 12 hr after the drug dose. In contrast to results obtained with the other neoplasms, chloropurine did not prevent the synthesis of polynucleotide guanine *de novo*, but did produce approximately 40 per cent retardation of glycine utilization for nucleic acid

adenine when the purine analog was administered between 1 and 12 hr before glycine-2-^{14}C.

Table 4 shows the data obtained using another tumor insensitive to the drug combination, the Mecca lymphosarcoma. Administration of glycine-2-^{14}C 1 hr after a dose of azaserine or chloropurine resulted in considerably less inhibition of isotope incorporation than was found with the sensitive neoplasms.

TABLE 3. THE BIOSYNTHESIS OF PURINES *de novo* IN 6C3HED LYMPHOSARCOMA ASCITES CELLS TREATED WITH AZASERINE OR CHLOROPURINE

Drug	Hours after drug dose	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Azaserine	0	8·7±0·7*	18·5±1·7	242·2±17·9
	1	0·4±0·1	0·2±0·1	4·9± 2·3
	3	0·7±0·2	0·3±0·06	5·4± 0·6
	6	1·5±0·3	1·5±0·8	19·6± 4·9
	12	3·0±1·2	6·5±2·5	38·1±11·9
Chloropurine	0	10·0±1·2	17·2±2·8	227·0±27·5
	5 (min)	9·9±1·2	19·0±4·0	269·8±20·4
	1	8·3±1·4	9·0±1·2	182·7± 7·7
	3	7·7±0·7	10·3±1·7	202·8±22·9
	6	9·8±1·8	10·1±1·2	170·6±26·4
	12	8·2±1·3	9·1±0·5	163·2± 2·4

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with either 0·2 mg azaserine per kg or 40 mg chloropurine per kg. At the designated time, 100 μg glycine-2-^{14}C (10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time point represents the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive glycine.

TABLE 4. THE BIOSYNTHESIS OF PURINES *de novo* IN MECCA LYMPHOSARCOMA ASCITES CELLS TREATED WITH AZASERINE OR CHLOROPURINE

Drug	Counts/min per μmole $\times 10^{-2}$		
	NA guanine	NA adenine	AS adenine
Control	1·6±0·1*	1·6±0·2	28·7±3·0
Azaserine	0·9±0·1	0·6±0·09	2·8±0·4
Chloropurine	1·2±0·5	1·6±0·4	27·2±5·2

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with either 0·2 mg azaserine per kg or 40 mg chloropurine per kg. One hour later, 100 μg glycine-2-^{14}C (10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. Control mice received an injection of isotonic saline 1 hr before radioactive glycine.

The results of experiments carried out with hepatoma 134 are given in Table 5. This neoplasm is susceptible to azaserine treatments; however, addition of chloropurine to this regimen did not elicit a further therapeutic response. Both azaserine

and chloropurine produced metabolic blocks of a magnitude and a duration comparable to those found using sarcoma 180 or Ehrlich carcinoma.

Chloropurine was without effect on the incorporation of glycine-2-^{14}C into residual protein in all of the neoplasms studied.

TABLE 5. THE BIOSYNTHESIS OF PURINES *de novo* IN HEPATOMA 134 ASCITES CELLS TREATED WITH AZASERINE OR CHLOROPURINE

Drug	Hours after drug dose	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Azaserine	0	4·2±1·0*	4·9±1·0	134·8±16·9
	1	0·4±0·06	0·5±0·1	16·2± 4·2
	12	0·5±0·1	0·8±0·1	30·3± 4·4
	24	1·3±0·4	1·7±0·4	45·4±10·4
Chloropurine	0	2·0±0·1	4·3±0·3	110·6± 8·8
	5 (min)	1·2±0·1	5·2±0·3	129·4± 9·3
	1	0·7±0·07	3·7±0·3	95·9± 7·3
	6	1·3±0·1	4·5±0·3	128·2± 8·8

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with either 0·2 mg azaserine per kg or 40 mg chloropurine per kg. At the designated time, 100 μg glycine-2-^{14}C (10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time point represents the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive glycine.

DISCUSSION

Measurements of the rates of incorporation of various isotopic precursors into polynucleotide purines after chloropurine treatment have indicated that this analog produced a metabolic block on the route to nucleic acid guanine formation *de novo*. The conversion of 5-amino-4-imidazolecarboxamide ribonucleotide to inosine 5′-phosphate (IMP) or the conversion of IMP to guanosine 5′-phosphate was considered to be the probable site of the sensitive enzyme.[11, 12] The major site of action of azaserine on this pathway is the conversion of α-N-formylglycinamide ribonucleotide to α-N-formylglycinamidine ribonucleotide.[4, 7–10]

Measurements of the magnitude and duration of the blocks produced by chloropurine and azaserine were made in a spectrum of ascitic neoplasms exhibiting differences in susceptibility to these agents in order to determine whether these blocks were associated with the carcinostatic properties of the drug combination. The results obtained are presented in Table 6. Data pertaining to inhibition of tumor growth are summarized from earlier experiments.[2] Azaserine prolonged the survival time of mice bearing Ehrlich carcinoma or sarcoma 180, while chloropurine was inactive. Combination of these agents in the therapy of these malignancies resulted in enhanced antitumor properties. In these two tumors, both azaserine and chloropurine produced metabolic blocks on the pathway to purine formation *de novo*. The 6C3HED lymphosarcoma showed some sensitivity to azaserine. This was in accord with the biochemical inhibition of purine biosynthesis by this antibiotic. The inability of

chloropurine to enhance the action of azaserine correlated with the lack of inhibition of glycine-^{14}C incorporation into nucleic acid guanine by the purine analog. This lack of inhibition did not appear to be due to cellular permeability, for chloropurine caused a retardation of radioactive glycine utilization for polynucleotide adenine. The Mecca lymphosarcoma was relatively resistant to drug therapy. This appeared to be due to the inability of both drugs to produce blocks of a magnitude found in the sensitive neoplasms.

Hepatoma 134 proved to be an exception. The growth of these cells was inhibited by azaserine; addition of chloropurine did not elicit further therapeutic response. However, biochemically each agent accomplished the metabolic blocks measured in a magnitude comparable to those found in the sensitive Ehrlich carcinoma and sarcoma 180. These results suggest that either the chloropurine and azaserine blocks which

TABLE 6. SUMMARY OF EFFECTS OF AZASERINE AND CHLOROPURINE ON SENSITIVE AND NATURALLY RESISTANT ASCITES TUMORS

Neoplasm	Inhibition of tumor growth*			Biochemical inhibition†	
	Azaserine	Chloropurine	Azaserine + chloropurine	Synthesis of purines *de novo* (azaserine)	Glycine-2-^{14}C into NA guanine (chloropurine)
Sarcoma 180	+	−	++	+	+
Ehrlich	+	−	++	+	+
6C3HED	+	−	+	+	−
Mecca	−	−	−	±	±
Hepatoma 134	+	−	+	+	+

* Degree of inhibition of ascites tumor growth as measured by prolongation of survival time: − indicates no effect; + indicates significant prolongation of survival; ++ indicates enhanced anti-tumor properties. Inhibition of 6C3HED was obtained using a measurement of the total packed cell volume.

† Degree of biochemical inhibition: − indicates no inhibition; + indicates a minimum of 90 per cent inhibition with azaserine and a minimum of 60 per cent inhibition with chloropurine.

were measured were unrelated to the growth inhibition by the drug combination, or that they were only part of the reason for the enhanced antineoplastic properties. However, it was also conceivable that the lack of correlation exhibited by hepatoma 134 was due to differences in the relative importance of alternate metabolic routes. This possibility was investigated in the following paper[14], using a number of preformed purines.

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