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Combination Chemotherapy of Cancer: Potentiation of Carcinostatic Activity of 8-Azaguanine by 6-Formylpteridine.* (19962)

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(Introduced by David V. Habif.)

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In a previous publication(1), it was reported that combination therapy with folic acid and 8-azaguanine produced greater carcinostasis than did therapy using 8-azaguanine alone. Folic acid itself caused no inhibition of the growth of the 755 mouse breast carcinoma used in these experiments. Insight into the mechanism of action whereby the carcinostatic activity of 8-azaguanine is augmented by folic acid was obtained through the observation that mammalian tissues contain enzymes capable of deaminating 8-azaguanine to 8-azaxanthine(2-4). Unpublished experiments evaluating the latter compound against tumor growth revealed this deaminated product of 8-azaguanine to be inactive as a carcinostatic agent. These initial observations have recently been confirmed and extended (4).

It was therefore hypothesized that folic acid or its degradation product 2-amino-4-hydroxy-6-formylpteridine (6-formylpteridine) inhibited the enzymatic deamination of 8-azaguanine *in vivo*, preventing the conversion of this carcinostatic agent into the non-carcinostatic compound, 8-azaxanthine(1). *In vitro* demonstration of the inhibitory effect of folic acid upon the enzymatic deamination of guanine and 8-azaguanine has been obtained (2). Evidence is presented in this report that (a) 6-formylpteridine augments the carcinostatic activity of 8-azaguanine, and (b) that this compound inhibits the enzymatic deamination of 8-azaguanine by tumor extracts.

Experimental. In vivo studies. The 755 tumor, a mammary adenocarcinoma, was subcutaneously transplanted to the axillary region of C_{57} black male mice by the usual trocar technic. The mice, weighing 18-25 g, and from 2 to 3 months of age received an *ad libitum* diet of Rockland pellets and water. The preparation of the 8-azaguanine for administration, its consistent carcinostatic effect upon the 755 tumor, the details of tumor growth measurement, and the method of statistical analysis have been previously described(1,5). 6-Formylpteridine, in weak alkaline solution, was injected intraperitoneally at various time intervals in relation to 8-azaguanine.

In vitro studies. The transplanted tumors (755) grown in C_{57} black mice, were removed immediately after killing the animals with ether, and stored for 24 or 48 hours in the frozen state until used. Pooled tumor tissue was homogenized for 3-5 minutes in a glass homogenizer of the Potter type with ice cold 0.1 M borate buffer at pH 8.4. The homogenates were then centrifuged at 22,000 g for 20 minutes in the cold. The resulting clear, pink, supernatant fluid at a concentration equivalent to 30% fresh tissue was used as the enzyme source. All operations were carried out at 4°C. 8-Azaguanine solutions were prepared as described previously(2) except that the

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TABLE I. Effect of 6-Formylpteridine Alone and in Combination with 8-Azaguanine on the 755 Carcinoma in C57 Male Mice.

Exp. No.	Group	Dose,* mg/kg	Time interval, hr†	Mean tumor wt, mg	No. of animals, dead/total	% change in body wt
38	Aza.			2049	3/20	+ 6
	Aza. + 6-FP	20	Simult.	1614	1/20	— 5
		20	1	1661	4/20	— 6
		20	½	1245	2/19	— 7
56	Control			863	0/19	+11
	6-FP	20		833	2/20	—13
	Aza.			438	0/20	+ 1
	Aza. + 6-FP	20	Simult.	488	1/20	— 2
		20	½	230	0/20	— 5
		20	1	265	0/18	— 3
58	Aza.			321	0/17	+ 3
	Aza. + 6-FP	15	½	182	0/17	— 4
		20	½	225	0/17	— 6
		20	1	281	0/18	— 3
64	Aza.			737	0/19	+ 1
	Aza. + 6-FP	20	½	358	0/20	— 6
		10	½	563	0/20	— 2
		10	¼	596	0/20	— 2
66	Control			1712	1/10	+13
	6-FP	20		1390	2/10	+ 4

* Refers to dose of 6-Formylpteridine. 8-Azaguanine always inj. at a dose of 50 mg/kg.
† 6-Formylpteridine inj. prior to 8-Azaguanine.
Daily intraper. therapy was begun upon well established tumors varying between experiments from 4 to 7 days old. The duration of tumor growth varied between experiments from 21 to 27 days.

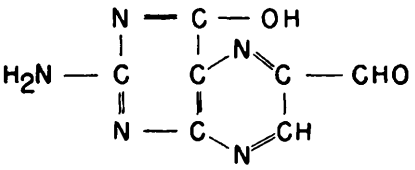
solvent used was 0.1 M borate buffer at pH 8.4. 6-Formylpteridine solutions were prepared similarly. The method used for following the deamination of 8-azaguanine by tumor extracts is based on a microdetermination of the ammonia nitrogen evolved during enzyme action(2,6).

Results and discussion. The *in vivo* effect of 6-formylpteridine alone and in combination with 8-azaguanine upon the 755 adenocarcinoma grown in the C_{57} male mice is recorded in Table I. Administered alone, 6-formylpteridine had no influence upon the growth of the neoplasm. The results obtained by the combination of 6-formylpteridine and 8-azaguanine depended upon the time interval between the injection of these 2 drugs. Negative results were consistently obtained if the 2 compounds were administered simultaneously. Statistically significant augmentation of the carcinostatic activity of 8-azaguanine occurred when the 6-formylpteridine was given a half hour prior to the injection of 8-azaguanine. Variable results, ranging from negative to almost significant enhancement of the inhibitory effect of 8-azaguanine upon tumor growth, ensued when the time interval was extended to one hour. Dosages of 6-formylpteridine less than 15 mg per kg body weight produced negative results regardless of the time interval. Only mild toxicity evidenced

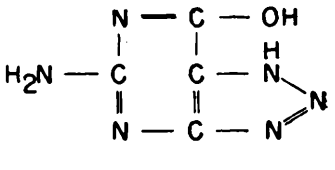
TABLE II. *In Vitro* Inhibition of Tumor Guanase by 6-Formylpteridine.*

6-Formylpteridine moles/ml reaction mixture	μg NH_2-N/g tissue wet wt present after 2 hr incubation with substrate at 37°C†	
	Pre-incubated‡	No pre-incubation§
.000	336	
.001	227	
.006	128	
.011	68	261
.016	41	

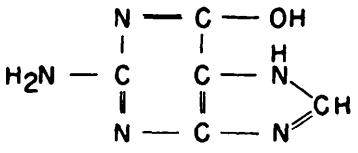
* 755 mammary adenocarcinoma grown in C57 mice. 8-Azaguanine was added in all cases to a level of .011 mole/ml of reaction mixture. Each reaction vessel contained $\cong$ 300 mg tissue, wet wt.
† Corrected for endogenous ammonia.
‡ Enzyme incubated 30 min. with 6-Formylpteridine before addition of 8-Azaguanine.
§ 6-Formylpteridine and 8-Azaguanine added simultaneously.



6-FORMYLPTERIDINE



8-AZAGUANINE



GUANINE

FIG. 1.

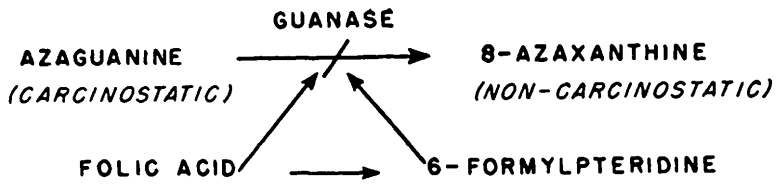


FIG. 2.

by slight loss in weight was observed in the group of animals receiving the drug combination.

6-Formylpteridine was observed to inhibit the *in vitro* deamination of 8-azaguanine by tumor extracts (Table II). This effect was most evident when the 6-formylpteridine and tissue extracts were incubated together for a 30-minute period prior to the addition of the 8-azaguanine. Simultaneous addition of inhibitor and substrate produced only slight inhibition. It is of interest that 6-formylpteridine, the primary photolytic decomposition product of folic acid(7,8), although structurally similar to both 8-azaguanine and guanine (Fig. 1), is itself not deaminated.

The lag in inhibition observed when 6-formylpteridine and 8-azaguanine were added simultaneously is of prime importance since it would seem to give insight into the *in vivo* phenomenon regarding the time interval necessary between 6-formylpteridine and subsequent 8-azaguanine administration for optimum augmentation of the carcinostatic effect of the latter compound. That a time interval is essential *in vitro* as well as *in vivo* tends to indicate that the observed effect is due either to a slow combination of the enzyme with inhibitor or to a further enzymatic modification of the molecule to one which is the true inhibitor. It seems unlikely that the necessity for a time interval is explained on the basis of differential tissue absorption of the 2 drugs.

The *in vitro* studies presented, although preliminary in nature, indicate that 6-formylpteridine can act as an inhibitor of 8-azaguanine deamination, thereby blocking the detoxification of 8-azaguanine and leaving more of this carcinostatic agent available at the tumor site (Fig. 2). The *in vivo* data demonstrating potentiation of the carcinostatic activity of 8-azaguanine by 6-formylpteridine supports this explanation. Such an hypothesis is given additional support by recent work demonstrating that differences in tumor guanase activity may account for the susceptibility or non-susceptibility of tumors to inhibition by 8-azaguanine(4).

These observations emphasize the importance of time-dosage studies not only in regard to fundamental mechanism studies, but also in the more practical application of combination chemotherapy.

Summary. 1. 2-Amino-4-hydroxy-6-formyl-

pteridine (6-formylpteridine), although non-carcinostatic by itself, augmented the carcinostatic action of 8-azaguanine against a mammary adenocarcinoma. This effect was found to be dependent upon a time interval between the injection of 6-formylpteridine and 8-azaguanine. 2. 6-Formylpteridine inhibited the *in vitro* deamination of 8-azaguanine by tumor extracts. Pre-incubation of enzyme with inhibitor was found to be essential for maximal inhibition. 3. It was postulated that the enhanced carcinostatic effect observed *in vivo* could be due to inhibition of 8-azaguanine deamination by 6-formylpteridine, since the deaminated product (8-azaxanthine) is non-carcinostatic.

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Influence of Cortisone on Nucleic Acids and Protein Content of the Chick Embryo. (19963)

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It has been demonstrated by Karnofsky *et al.*(1) that cortisone inhibits both the somatic development and the growth of the chick embryo. In view of the relationship existing between nucleic acid metabolism, protein synthesis and growth, it seemed interesting to study whether this inhibitory effect of cortisone on growth is associated with some change in the ribonucleic (RNA), desoxyribonucleic (DNA) acids and in the protein nitrogen (N) content of the chick embryo.

Methods. Fertile White Leghorn eggs purchased on the market were incubated at 38°C and 75% relative humidity. At 8 days of development, the chorioallantoic membrane was injected with 1.25 mg of cortisone acetate (Merck) per egg; controls were similarly treated with the saline suspension alone. The surviving embryos were sacrificed starting from the 11th to the 18th day of incubation, *i.e.,* from 3 to 10 days after the treatment, 4 embryos per group per day. They were weighed after freezing, then transferred to a Waring Blendor. The homogenate was analyzed for protein N content (precipitation with 14% trichloracetic acid and micro-Kjeldahl determination) and for DNA and RNA content by the method of Schmidt-Thannhauser(2).

Results. In the cortisone-treated embryos, growth and somatic differentiation were clearly inhibited starting from the third day after hormone injection; the effect being more evident in the following days. Fig. 1 presents the mean body weight and the analytical data in control and cortisone treated embryos; the protein N and the DNA and RNA values are plotted as mg of N and as μg of P, respectively. It appears that corti-