Biochemical Pharmacology, 1961, Vol. 7, pp. 187–194. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF PURINE ANTAGONISTS ON THE METABOLISM OF OTHER PURINE ANTAGONISTS

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(*Received* 5 *January* 1961)

Abstract—The effects of azaserine, thioguanine and 6-mercaptopurine on the metabolism of 4-aminopyrazolopyrimidine-^{14}C and of 4-aminopyrazolopyrimidine on the metabolism of thioguanine-^{14}C and of 6-mercaptopurine-^{14}C have been studied in Ehrlich ascites carcinoma and ascites sarcoma-180. Azaserine, thioguanine and 6-mercaptopurine not only enhanced the inhibition of tumor growth by 4-aminopyrazolopyrimidine, but also increased the amount of incorporation of the analog into tumor polynucleotides. Enhancement of the size of the pool of 4-aminopyrazolopyrimidine mononucloetides was evident during dual treatment with azaserine and thioguanine. The amount of incorporation of radioactivity from labeled 6-mercaptopurine into polynucleotides was decreased by 4-aminopyrazolopyrimidine, while little change in the size of the labeled mononucleotide pool was evident. The therapeutic effectiveness of thioguanine was increased by the co-administration of 4-aminopyrazolopyrimidine and the amount of incorporation of thioguanine into nucleic acids was somewhat increased by 4-aminopyrazolopyrimidine. These results are discussed with respect to present hypotheses concerning the anabolic conversion of purine analogs into their active derivatives.

POTENTIATION of carcinostatic activity has been observed when several different purine analogs have been used in combination. The pairs, 4-aminopyrazolopyrimidine (APP) plus 6-mercaptopurine (6-MP) and APP plus thioguanine (TG), show potentiation in Ehrlich ascites carcinoma (Ehrlich) and in ascites sarcoma-180 (S-180), even though APP alone is not active in sarcoma-180.[1] Combination therapy of Ehrlich carcinoma with TG plus 6-MP also shows potentiation.[2] In addition, combinations of azaserine with APP,[1] TG,[5, 6] 6-chloropurine,[5, 7] 8-azaguanine,[3] and 6-MP[3, 8, 9] have also been shown to potentiate carcinostasis in a variety of tumors. Other reports of combination chemotherapy of tumors have been reviewed by Goldin and Mantel[4].

The mechanism of action of none of these purine antimetabolites is currently known with certainty. While the primary site of azaserine action in mammalian tissues is accepted as inhibition of the biosynthesis of purines *de novo*,[10] there remain secondary aspects of the action of this drug which are unclear.[11–13] Thus, it is difficult if not impossible to ascertain exactly the mechanisms by which combinations of these agents potentiate carcinostasis. However, this phenomenon offers contributory evidence that the drugs used in combination have different mechanisms of action, for otherwise only additive effects would be expected.

One possible factor in potentiation which can readily be investigated is the effect of one drug on the metabolism of the other. Because the active forms of many of the

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purine antimetabolites are believed to be their ribonucleotides,[14-16] it seemed useful to look for alterations in the concentrations of the ribonucleotides of the drugs in the umor cells during potentiation. In two cases of potentiation with azaserine changes of this sort have been shown to occur. LePage[12] found that a TG-resistant subline of the Ehrlich carcinoma could be restored to sensitivity in a potentiative manner when treated with both TG and azaserine, and that the concentration of the TG-ribonucleotide and the incorporation of TG into nucleic acids increased concomitantly. Paterson[13] also observed increased formation of 6-MP ribonucleotide following co-administration of azaserine. However, studies of the simultaneous metabolism of two purine analogs, when used in combination therapy, have not been reported previously.

Biochemical and biological studies of the action and the metabolism of the adenine analog, APP, have recently been made in this laboratory.[1, 15] Combinations of this compound with 6-MP, TG or azaserine potentiated carcinostasis. Therefore it was of interest to determine the effect of these drugs on the metabolism of APP in two mouse tumors, one sensitive to the antimetabolite when used alone (Ehrlich ascites carcinoma), the other sensitive only in combination therapy (ascites sarcoma-180). To complete the study, the influence of APP on the metabolism of 6-MP and of TG in the same tumors was also studied.

METHODS

4-Aminopyrazolopyrimidine-6-^{14}C was a gift of Dr. L. L. Bennett, Jr., Southern Research Institute, Birmingham, Ala., to whom we are very grateful. The compound was subjected to further purification in this laboratory and when used contained less than 1 per cent impurity. As measured, this material had a specific activity of 2000 counts/min per μg. 6-Mercaptopurine-8-^{14}C was purchased from Isotope Specialties Co., Inc., and diluted with non-radioactive material until its specific activity was 475 counts/min per μg. Thioguanine-8-^{14}C was synthesized by Dr. G. A. LePage of this laboratory. His gift of this material, which had a specific activity of 2600 counts/min per μg, is greatly appreciated. Azaserine and non-radioactive APP were obtained from the Cancer Chemotherapy National Service Center, National Cancer Institute; 6-MP from the California Corporation for Biochemical Research; and the TG was synthesized in this laboratory. All compounds were dissolved in 0·154 M sodium chloride before use.

Female Swiss mice, 25–30 g, were inoculated with 1×10^6 Ehrlich ascites carcinoma cells or 6×10^6 sarcoma-180 ascites cells, and used 6 days thereafter.

Ascites tumor cells were removed from the mice by capillary pipette after laporotomy, separated from the ascitic fluid by centrifugation, and extracted three times with cold 0·2 M perchloric acid. The combined extracts were neutralized with potassium hydroxide, chilled and the potassium perchlorate was removed. The acid-insoluble residue was suspended in 0·4 M perchloric acid and heated at 100 °C for 30 min. This hot extract was combined with one 0·4 M perchloric acid-washing of the precipitate, neutralized with potassium hydroxide, the potassium perchlorate was removed in the cold, and the solution was evaporated to dryness. Each sample was redissolved in 1 ml of 0·05 M hydrochloric acid and an aliquot was taken for radioactivity measurements in a micro-thin window gas-flow counter. The radioactivity in this fraction was used as a measure of incorporation of the analogs into the combined nucleic acids.

Metabolites of APP-^{14}C were separated from the neutralized cold acid extracts by chromatography on 10×40 mm Dowex-1-formate columns. The effluent, plus 10 ml

of water, contained the free base and ribonucleoside, which were not separated because it had previously been found that the ribonucleoside usually comprised only about 10 per cent of this fraction.[15] To the column were added 15 ml of 0·05 M formic acid to elute the degradation products of APP, which consisted of 4-hydroxypyrazolopyrimidine plus several unidentified compounds;[15] 15 ml of 2·0 M formic acid to elute the ribonucleoside monophosphate of this drug; 15 ml of 4·0 M formic acid containing 0·2 M ammonium formate to elute the ribonucleoside diphosphate; and 30 ml of 2·0 M formic acid containing 2·5 M ammonium formate to elute the ribonucleoside triphosphate. Further details of the separation and identification of these metabolites have been presented previously.[15]

Metabolites of TG-^{14}C and of 6-MP-^{14}C were separated by a manual chromatographic procedure based on the gradient elution chromatographic separations of these compounds by Moore and LePage[16] and by Paterson[13]. Neutralized tissue extracts were poured on to 10 × 40 mm Dowex-1-formate columns. The effluent, plus 15 ml of 0·05 M formic acid, contained the free bases and ribonucleosides of these compounds, which were not further separated. Fifteen milliliters of 3·0 M formic acid eluted the degradation products, 6-thio-uric acid and 6-thio-xanthine, which were not further separated. Thirty milliliters of 2·0 M formic acid containing 2·5 M ammonium formate eluted the ribonucleotides of both TG and 6-MP.

Each fraction was evaporated to dryness and ammonium formate, if present, was sublimed off. Each sample was redissolved in 1 ml of 0·05 N hydrochloric acid and an aliquot was taken for radioactivity measurements.

RESULTS

To determine the effects of one drug on the metabolism of the other, both drugs of the pair under consideration were simultaneously injected intraperitoneally into mice bearing 6-day tumors. One of each drug pair was labeled with radiocarbon. The following combinations were employed: APP-^{14}C plus azaserine; APP-^{14}C plus TG; APP-^{14}C plus 6-MP; TG-^{14}C plus APP; and 6-MP-^{14}C plus APP. The doses used in all cases were those employed in therapy: 750 μg of 6-MP; 200 μg of APP; 25 μg of TG; and 5 μg of azaserine.[1] One, two and four hours later the tumor cells were removed from these animals, extracted with perchloric acid, and the concentrations of radioactive drug metabolites determined as described above. All results are expressed as the averages of determinations from three to six mice analyzed separately, and the variation among samples averaged 10 per cent. Radioactivity is presented as microgram-equivalents of the injected free base per gram wet weight of tissue.

Table 1 shows the effect of azaserine on the metabolism of APP-^{14}C. The principal effect of azaserine was to increase the amount of ribonucleotides of APP found in these cells. This increase was greater in sarcoma-180 than in Ehrlich carcinoma, a difference which can be correlated with therapeutic response. The former tumor is not sensitive to APP alone, but becomes quite sensitive to combination treatment.[1] There was also an increase in the amount of incorporation of APP-^{14}C into the nucleic acid fraction at the early time periods. (This incorporation has previously been shown to be by true internucleotide linkage.[15])

The effect of TG on the metabolism of APP-^{14}C, as shown in Table 2, was somewhat similar to that exerted by azaserine. The amount of ribonucleotides of APP was consistently increased in Ehrlich carcinoma after TG co-administration. In sarcoma-180

TABLE 1. EFFECT OF AZASERINE ON THE METABOLISM OF APP-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	1·87	1·78	1·12	26·9	23·8	18·7	0·204	0·242	0·097	1·96	1·73	0·596
	+ azaserine	3·50	1·91	1·23	31·5	29·2	20·1	0·515	0·150	0·144	1·96	1·55	0·382
S-180	control	1·32	1·08	0·994	21·2	20·6	20·0	0·204	0·149	0·161	0·997	0·911	0·661
	+ azaserine	1·86	1·52	0·981	32·1	27·8	22·3	0·246	0·114	0·086	1·54	1·43	0·554

* Tumor-bearing mice were each injected with 200 μg of APP-^{14}C and 5 μg of azaserine.

TABLE 2. EFFECT OF TG ON THE METABOLISM OF APP-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	1·87	1·78	1·12	26·9	23·8	18·7	0·204	0·242	0·097	1·96	1·73	0·596
	+ TG	3·49	3·83	2·38	31·8	26·2	22·6	0·404	0·538	1·69	1·77	1·29	0·671
S-180	control	1·32	1·08	0·994	21·2	20·6	20·0	0·204	0·149	0·161	0·977	0·911	0·661
	+ TG	2·91	1·37	0·663	36·4	19·1	15·8	0·528	0·399	0·112	2·01	0·746	0·687

* Tumor-bearing mice were each injected with 200 μg of APP-^{14}C and 25 μg of TG.

TABLE 3. EFFECT OF 6-MP ON THE METABOLISM OF APP-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	1·87	1·78	1·12	26·9	23·8	18·7	0·204	0·242	0·097	1·96	1·73	0·596
	+ MP	4·99	2·00	1·51	25·8	20·8	17·1	0·320	0·183	0·100	1·26	1·18	0·444
S-180	control	1·32	1·08	0·994	21·2	20·6	20·0	0·204	0·149	0·161	0·977	0·911	0·661
	+ MP	4·02	2·75	1·70	21·9	17·9	13·1	0·211	0·290	0·559	1·40	1·15	0·631

* Tumor-bearing mice each received 200 μg of APP-^{14}C and 750 μg of 6-MP.

TABLE 4. EFFECT OF APP ON THE METABOLISM OF TG-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	2·64	0·907	0·632	4·09	2·63	1·75	3·77	1·32	1·10	3·32	1·18	0·835
	+ APP	3·16	1·58	0·892	3·59	2·42	1·92	2·43	0·809	0·602	3·42	1·08	0·906
S-180	control	1·01	0·900	1·18	2·89	2·22	1·88	5·45	2·36	2·80	1·92	1·93	1·98
	+ APP	5·88	2·40	1·16	2·78	2·28	2·00	1·83	1·49	1·10	1·19	1·36	1·32

* Tumor-bearing mice each received 25 μg of TG-^{14}C and 200 μg of APP.

TABLE 5. EFFECT OF APP ON THE METABOLISM OF 6-MP-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	1·15	1·09	0·940	4·92	4·86	2·94	0·232	0·363	0·200	1·34	1·84	1·62
	+ APP	0·900	0·717	0·950	4·62	3·80	2·28	0·224	0·303	0·220	1·10	1·41	1·35
S-180	control	0·565	0·758	0·364	5·07	5·16	2·20	0·237	0·297	0·220	1·45	2·18	1·59
	+ APP	0·520	0·336	0·439	4·71	2·77	2·53	0·225	0·310	0·159	2·04	1·03	1·07

* Tumor-bearing mice were each injected with 750 μg of 6-MP-^{14}C and 200 μg of APP.

increased ribonucleotide levels were noted at the first hour, but in this tumor the concentration of the ribonucleotide of the analog decreased more quickly than in the controls, until it was significantly less than control values at 4 hr. Increased amounts of incorporation into nucleic acids were also evident in Ehrlich carcinoma throughout the time course studied, and in sarcoma-180 at the first hour.

Table 3 shows the effect of 6-MP on the metabolism of APP-^{14}C, which differed from that of azaserine or TG. While the amount of incorporation of the radioactive drug into the nucleic acids was increased by the co-administration of 6-MP, the acid-soluble ribonucleotide concentration in all cases was equal to or less than the control values. Other fractions were only slightly changed.

To complete this study, the metabolism of the two other purines involved both in potentiation of carcinostasis and alterations in the metabolism of APP was investigated. Table 4 shows the effect of APP on the metabolism of TG-^{14}C in these tumors. More TG was incorporated into nucleic acids and less into acid-soluble ribonucleotides following the co-administration of APP than in the control cells. While little or no change in the amount of degradation products was observed, the content of free base of TG was lowered after combination therapy. In this case it appears that the total amount of TG present in the APP-treated cells was less than in the controls. (It might be pointed out that very little desulfuration of TG occurs in these tumors,[11] so radioactivity can be equated to drug content with considerable reliability.)

Finally, the effect of APP on the metabolism of 6-MP-^{14}C was studied (Table 5). The amount of incorporation of radioactivity from 6-MP-^{14}C into the tumor nucleic acids was decreased in some cases by the co-administration of APP, while the concentrations of acid-soluble ribonucleotides of 6-MP were slightly below the control values. (While it has been shown that most of the radioactivity in these tumors really is associated with 6-MP and its metabolites,[13] some desulfuration does occur. It is particularly likely to be the case that much of the radioactivity in the nucleic acid fraction was contained in adenine and guanine.[17, 18])

DISCUSSION

It is evident from the results presented that two purine antimetabolites may be metabolized differently in the presence of each other than each is by itself, and that different tumors vary in the extent to which this happens. In the cases observed, however, the alterations have all been quantitative, rather than qualitative, and on the whole have not been large. The most notable and consistent changes were in two fractions: the concentration of acid-soluble ribonucleotides of the drugs (their presumptive active forms), and the amount of ribonucleotide incorporated into the nucleic acids. Increases were noted in the amounts incorporated into nucleic acid, while both increases and decreases were found in acid-soluble ribonucleotide concentrations. Thus, the concentrations of the ribonucleotides of the potentiating drugs did not always parallel their increased carcinostatic activity. This appears to be an exception to the commonly accepted concept which relates the amount of ribonucleotide formation by purine analogs to the extent of tumor inhibition.[14–16]

Two general mechanisms may be adduced to explain such phenomena. The two drugs and their metabolites may compete for enzymes and co-factors involved in their metabolism; or, more likely, these changes in metabolism may be the direct results of the actions of both drugs on the purine metabolism of the cells, each drug probably having

a different action. For example, analogs may compete either for pyrophosphorylases involved in their conversion to nucleotides, or for degradative enzymes. Also, a drug which may affect one area of purine metabolism may indirectly make its companion drug more or less susceptible to the action of the enzymes concerned. The thirty-fold range in the doses of the purine analogs used also may influence the effects observed.

Azaserine is a special case. It is an irreversible inhibitor the action of which depends only on those molecules which attach to the proper enzyme.[10, 19] This happens within minutes after its injection and it seems improbable that the purine analogs interfere with this process. Whatever effect the purines might have on excess azaserine molecules, it seems unlikely that the carcinostatic process would be affected. Since azaserine at the dose used affects purine metabolism directly only by interruption of the *de novo*-biosynthesis of purines,[20] it is suggested that its effects on the metabolism of APP (and that of TG and 6-MP as well[12, 13]) are caused by perturbations in the metabolism of purine ribonucleotides resulting from inhibition of *de novo*-biosynthesis, or by some effect of the formylglycineamide ribonucleotide which accumulates during treatment with azaserine.

Thus, the mechanisms by which these alterations in drug metabolism come about remain unclear, as are the processes by which these drug combinations potentiate carcinostasis. One factor probably is the increase in the amount of the ribonucleotides of the analogs which is present in the cells after treatment with two drugs, as compared to that after only one, but, as pointed out above, this is not a complete explanation. It is also conceivable that the disruptive action of one drug may make certain aspects of purine metabolism unusually sensitive to inhibition by the other analog.

Acknowledgements—This work was supported by Contract no. SA-43-ph-3068 with the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, and by grants from the U.S. Public Health Service (CY-4551) and the Gustavus and Louise Pfeiffer Research Foundation.

The authors wish to express their appreciation to Dr. G. A. LePage, in whose laboratory this work was done, and to acknowledge the assistance of Miss Noel Howard in making the radioactivity measurements.

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