Arabinosyl-6-mercaptopurine and Arabinosyl-6-mercaptopurine-5'-phosphate: Comparison of Their Metabolic Effects* (34035)

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In a series of studies on arabinosyl-6-mercaptopurine (Ara-6-MP),[1] this nucleoside analog was found to be carcinostatic for a number of experimental tumors (1–3). Although it is a purine nucleoside, the only metabolic effect observed in susceptible tumor cells was an inhibition of the cytidine phosphate reductase. Inhibition of bone marrow growth in mice, as indicated by white blood cell counts, could only be obtained by the use of very high and frequent dosages (3). Excretion in the urine was almost entirely as unchanged Ara-6-MP. No indication was obtained that either cleavage to 6-mercaptopurine (6-MP) or conversion to nucleotide occurred. This is in contrast to other purine and pyrimidine analogs that require conversion to nucleotides as an activation process (4–6). We therefore have carefully reexamined the question as to whether the inhibitory effects of Ara-6-MP could be explained by the formation of minute amounts of nucleotide(s) of high activity. Tests with susceptible tumor cells were made *in vivo*, and *in vitro*, and in cellfree extracts of such tumors supported by a phosphate-generating system.

Methods and Materials. The Ara-6-MP was obtained from the Cancer Chemotherapy National Service Center. Ara-6-MPMP was synthesized as described earlier (7). Both were labeled with radiosulfur, by a procedure described for other nucleosides (8), and were recrystallized until essentially radiochemically pure. Adenosine triphosphate, creatine phosphate, and crystalline creatine phosphokinase were purchased from Sigma Chemical Co., St. Louis. Growth and maintenance of the tumor cell lines used (Ca 755, Ehrlich, L1210) has been described (9).

Cell-free extracts of ascites tumor cells were prepared by homogenizing in water at 0° for 1 min in a Virtis model 23, restoring toxicity with solid NaCl, homogenizing 1 min more, and centrifuging for 90 min at 16,000*g*. Protein content of the extracts, varying from 12 to 19 mg/ml, was determined by the method of Lowry *et al.* (10). Incubations to test for nucleotide formation were carried out at 37° in a 1.00-ml volume. The mixture contained 0.30 ml of extract; 0.5 μmoles of substrate; 1.58 μmoles of ATP; 15.8 μmoles of creatine phosphate; 118 μg of creatine phosphokinase; 79 μmoles of Tris buffer, pH 7.4, and 7.9 μmoles of $MgCl_2$. Reactions were stopped by chilling to 0° and adding 26% PCA to a final concentration of 2%. The precipitated proteins were centrifuged and the supernatant fluids were separated. KOH(2 *M*) was used to neutralize to pH 4.8. After $KClO_4$ had precipitated in the cold, aliquots were spotted on Whatman 3MM papers and developed by descending chromatography with isobutyric acid:concentrated NH_4OH:H_2O (66:1:33). The chromatograms were air-dried and cut into 1-cm strips, which were subjected to scintillation counting in a toluene fluor. The patterns of radioactivity were compared for samples incubated with and without cell-free extract.

The use of Ara-6-MPMP-^{35}S as a substrate provided a test for phosphomonesterase activity and probably for the formation of di- and triphosphates.

In vivo tests were made for formation of nucleotides from Ara-6-MP-^{35}S by L1210 ascites tumor cells and normal mouse tissues.

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[1] Abbreviations used: Ara-6-MP, 9-β-D-arabinofuranosyl-6-mercaptopurine; Ara-6-MPMP, β-D-arabinofuranosyl-6-mercaptopurine-5'-monophosphate; 6-MP, 6-mercaptopurine; 6-MeMPR, 6-methylmercaptopurine riboside; ATP, adenosine triphosphate; PCA, perchloric acid; TPNH, reduced triphosphopyridine nucleotide.

For example, an AKD2F1 female mouse bearing a 5-day growth of L1210 ascites cells and a normal Swiss female mouse were each given an intraperitoneal injection of Ara-6-MP^{35}S (50 mg/kg, 3.57 $\times$ 10^6 cpm/μmole). After 1 hr, each was sacrificed and the tissues were rapidly removed to dry ice, except for the L1210 cells, which were centrifuged and extracted at 0°. Each tissue was homogenized in 2% PCA at 0° and the extracts were quickly neutralized to pH 4.8 with 2 *M* KOH. These manipulations were shown to have no effect upon Ara-6-MPMP; *i.e.*, no cleavage to free base or nucleoside occurred. Aliquots of each tissue extract were spotted on 3 Whatman 3 MM papers and descending chromatography was conducted in three separate systems (a) isobutyric acid–ammonia–water (66:1:33), (b) 95% ethanol–sodium borate–5 *M* ammonium acetate (pH 9.5)–Versene (220:80:20:0.5), and (c) 5% Na_2HPO_4. The R_f values for Ara-6MPMP, Ara-6-MP, and 6-MP in system (b) were: 0.08, 0.50, 0.44, respectively. In system (c) they were: 0.84, 0.64, 0.35, respectively. In system (a) the solvent was run for 30 hr because of low mobilities of nucleotides. In this time the 6-MP was washed off the end; the Ara-6-MP moved well down the sheet ahead of Ara-6-MPMP. This system is known to separate mono-, di-, and triphosphates of similar nucleosides (8).

In vitro experiments with cell suspensions utilized Ehrlich ascites tumor cells grown for 5 days in Swiss female mice. These were washed from the peritoneal cavity and centrifuged 7 min at 1470*g*. Incubations were carried out at 37° in 50-ml erlenmyer flasks gassed with 95% O_2 – 5% CO_2. Each flask contained 8.0 ml of Robinson's salts–glucose–bicarbonate medium at pH 7.4 (11), with 0.50 ml of suspended ascites cells and 2.0 μmoles Ara-6-MP-^{35}S (8.25 $\times$ 10^6 Ci/min/μmole). Incubation was terminated by chilling the flasks in ice. The cell suspensions were centrifuged and the medium was discarded. The cells were extracted with 2.5 ml of 2% PCA, in each case, and centrifuged. The extracts were separated from the precipitates and neutralized to pH 4.8 with 2 *M* KOH. An aliquot of each extract equivalent to 10% of its volume was chromatographed on 3 MM paper with the ethanol–borate–ammonium acetate–versene system. The area of each chromogram corresponding to the Ara-6-MPMP was cut out and counted in a liquid scintillation system.

Cytidylate reductase assays were carried out with the same extracts used to test for nucleotide formation. The incubation mixture contained: cytidine-5′-diphosphate-2-^{14}C, (2.0 μCi/μmole, Schwarz Bioresearch Inc.), 100 mμmoles; ATP, 0.5 μmoles; $MgCl_2$, 1.5 μmoles; TPNH, 60 mμmoles; Tris buffer, pH 7.4, 50 μmoles; cell-free extract, 0.15 ml and Ara-6MP or Ara-6-MPMP as indicated. Incubations were at 37° in 0.60-ml volumes and were stopped by the addition to each of 1.00 ml of 10% PCA. Protein precipitates were removed in the centrifuge and the supernatant fluid was heated 10 min at 100° in order to convert di- and triphosphates to monophosphates. Then the solutions were neutralized to pH 7 with 5 *M* KOH. Each sample was run onto a 5 $\times$ 30-mm Dowex-50H column followed by 2–3-ml water washes and 5 ml of *M* acetic acid. The cytidine and deoxycytidine phosphates were then eluted with 7 ml of 1 *M* HCl. The HCl eluates were evaporated to dryness *in vacuo* in small beakers. Each residue was taken up in 0.30 ml of H_2O and 0.25 ml was spotted on Whatman 3MM paper. Papers were developed for 40 hr by descending chromatography with 95% ethanol–saturated sodium borate–5 *M* ammonium acetate, pH 9.5–ethylenediaminetetraacetate, 0.5 *M* (66:24:6:0.15). Cytidine, cytidine-5′-monophosphate and deoxycytidine-5′-monophosphate (Sigma Chemical Co.) were used as carriers. The deoxycytidine-5′-monophosphate spots were counted in a scintillation system as a measure of reductase activity.

Results. Although the cell-free extracts of the three mouse tumors tested readily converted 6-MeMPR-^{35}S to the nucleoside monophosphate, incubations with Ara-6-MP-^{35}S gave no detectable product. Incubations of cell-free extracts with Ara-6-MPMP-^{35}S gave a slow conversion to Ara-6-MP-^{35}S. This is

TABLE I. Kinase and Phosphomonoesterase Activities of Cell-Free Extracts from Mouse Tumors.[a]

Incubation time (min)[b]	Metabolite formed (mμmoles/mg of protein)					
	Ara-6-MPMP[c] → Ara-6-MP			6-MeMPR → 6-MeMPRP		
	Ca755	Ehrlich	L1210	Ca755	Ehrlich	L1210
5	0	0	0	2.1	10.0	1.3
15	0	5.5	0	4.8	28.3	2.0
30	0	2.2	4.5	12.4	53.0	3.5
60	3.0	7.8	8.0	26.7	112	5.4

[a] Results are averages of duplicate incubations in a single experiment.

[b] Incubation at 37°.

[c] Abbreviations: Ara-6-MP, β-arabinosyl-6-mercaptopurine; Ara-6-MPMP, β-arabinosyl-6-mercaptopurine-5′-monophosphate; 6-MeMPR, 6-methylmercaptopurine riboside; 6-MeMPRP, 6-methylmercaptopurine riboside-5′-monophosphate.

shown by data presented in Table I. No nucleotide could be detected in any of the incubations with Ara-6-MP-^{35}S. When Ara-6-MPMP-^{35}S was the substrate, no di- or triphosphate formation could be detected. A similar experiment, conducted with an extract of Ca755 cells, used inosine-8-^{14}C as a positive control. Again no nucleotide formation from Ara-6-MP could be detected, although inosine-8-^{14}C was converted to nucleotide at a rate of approximately 3 mμmoles/min/mg of protein.

The *in vitro* incubation of Ehrlich ascites cells, carried out for various lengths of time (15–60 min) gave no indication of nucleotide on the chromatograms, although xylosyladenine was converted to nucleotide to the extent of approximately 250 mμmoles/15 min. It was estimated that 0.2 mμmole of nucleotide from Ara-6-MP could have been detected.

In vivo tests for formation of nucleotides from Ara-6-MP^{35}S in mouse tissues, including the susceptible L1210, did not indicate any such product. It was estimated that as little as 2 mμmoles/g could have been detected.

The tests on cytidylate reductase, with the 5′-monophosphate of Ara-6-MP obtained by chemical synthesis, were conducted with Ara-6-MP as a positive control. Although much greater inhibition of the cytidilyate reductase would be obtained if the extracts were preincubated with Ara-6-MP (3), no preincubation was used in these experiments in order to minimize the possible compromising effects of conversion of Ara-6-MPMP to Ara-6-MP. If the nucleotide form were the active inhibitor, nucleotide would be expected to be effective at a lower concentration. As shown in Fig. 1, Ara-6-MP produced moderate and significant inhibitory effects at 5×10^{-5} *M*, after 20 min of incubation. Concentrations of Ara-6-MPMP from 5×10^{-6} to 5×10^{-5} *M* did not produce consistent or significant inhibitions. Tests on cytidylate reductase with what appeared to be the 2′, 3′, 5′ triphosphate of Ara-6-MP (7) showed that it was inactive. Since 5′-di- or triphosphate of Ara-6-MP could not be detected in incubations with Ara-6-MP, the chemical synthesis of these nucloetides was not attempted.

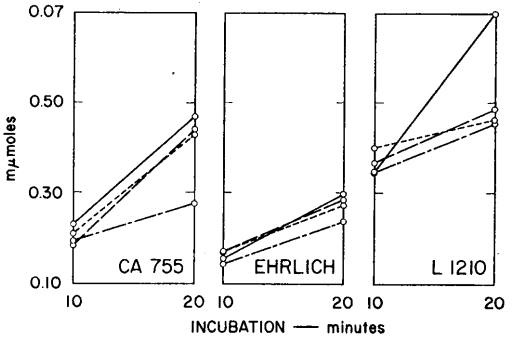

Fig. 1. Cytidylate reductase activity in extracts of ascites tumor cells; the influence of arabinosyl-6-mercaptopurine and arabinosyl-6-mercaptopurine-5′ -phosphate: (O—), control; (O— –), Ara-6-MP, 5×10^{-5} *M*; (O— —), Ara-6-MPMP, 5×10^{-5} *M*; (O-----), Ara--MPMP, 5×10^{-6}*M*.

Conclusion. It was found that less than 2 mμmoles of nucleotide/g could be formed *in vivo* in a susceptible tissue, that suspensions of susceptible ascites cells could form less than 0.4 mμmoles/g, and that fortified cell-free extracts of susceptible tumors could not form nucleotide, nor convert 5′-nucleotide to di- or triphosphate. This, along with the lack of inhibitory effects of synthetic Ara-6-MPMP in the cytidylate reductase system that was inhibited by Ara-6-MP leads us to conclude that Ara-6-MP, *i.e.*, the nucleoside as such, is the active inhibitor.

Summary. Arabinosyl-6-mercaptopurine (Ara-6-MP), an effective inhibitor of the growth of the mouse tumors Ca755, Ehrlich and L1210, was incubated in fortified cell-free extracts of these tumor cells under conditions where 6-methylmercaptopurine riboside and inosine were converted to nucleotide. No nucleotide formation could be detected. *In vivo* experiments with L1210 cells and normal mouse tissues also indicated that no nucleotide was formed. The 5′-phosphate of Ara-6-MP, obtained by chemical synthesis, had no apparent effect on the cytidylate reductase of tumor cell extracts under conditions such that Ara-6-MP was inhibitory. It was concluded that Ara-6-MP, a "fradulent nucleoside," is active as such and does not require conversion to nucleotide for activation.

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