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Feedback Inhibition by 6-Methylthioinosine 3′,5′-Cyclic Monophosphate in Tumor Cells Resistant to the Nucleoside[1] (39083)

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The development of drug resistance to fraudulent nucleosides used in cancer chemotherapy is well known. In general, cellular resistance to nucleoside analogs should occur via one of the following mechanisms. (1) The cellular membrane could conceivably become impermeable to the drug. (2) The analog could be rapidly inactivated by catabolic enzymes. (3) Activating enzymes could lose affinity for the analogs or activating enzyme activities could be lost by mutation or deletion. (4) Target enzymes could lose affinity for the activated nucleoside analogs. For instance, arabinosyladenine (ara-A) can be rapidly converted to the inactive arabinosylhypoxanthine in the target cell by adenosine deaminase (1). Loss of adenosine kinase activity confers cellular resistance to methylthioinosine (MTI)[2] (2, 3). The adenosine kinase of one tumor resistant to MTI was unimpaired in its ability to phosphorylate adenosine but was unable to convert MTI to MTIMP (3), suggesting enzyme loss of affinity for MTI. In addition, Henderson *et al.* (4) have observed that the first enzyme in the *de novo* purine pathway of a murine tumor line resistant to MTI had lost affinity for the nucleotide derivative. Recently, cyclic nucleotide analogs were shown to be effective as antitumor agents by LePage and Hersh (5), Hughes and Kimball (6), and Cho-Chung (7). Cyclic nucleotides would require conversion to the 5′-nucleotides by a phosphodiesterase(s) (6) in order to exert their activities. This would circumvent drug resistance due to loss of kinase activity, but, presumably, would not bypass drug resistance due to loss of affinity of the target enzyme for the nucleotide analog. LePage and Hersh (5) have reported previously that the 3′,5′-cyclic phosphate derivative of methylthioinosine (c-MTIMP) inhibited the growth of a tumor line resistant to methylthioinosine (MTI). In this report, we show that cMTIMP is converted to the 5′-nucleotide by a cyclic phosphodiesterase(s) and produces feedback inhibition of *de novo* purine nucleotide biosynthesis in a murine tumor resistant to MTI. In addition, we show that the resistant tumor cells still retain the capacity to be inhibited by adenosine but not by MTI which confirms the observation of Lomax and Henderson (3).

Materials and methods. Female Swice mice (Sprague–Dawley, Madison, Wis.) were each implanted with 2×10^6 Ehrlich ascites tumor cells either sensitive to (EAC/s) or resistant to MTI (EAC/MTI). The EAC/MTI ascites tumor cells were a gift from Dr. D. H. W. Ho, M. D. Anderson Hospital, Houston, Texas, and were maintained by injections of MTI, 10 mg/kg, given ip once daily for 3 consecutive days starting 24 hr after tumor implantation.

Enzyme assays. The assays for cyclic phosphodiesterase activity were performed according to the spectrophotometric method described in (6) and the procedure of Thompson and Appleman (8). The adenosine kinase assay is described in (6). All assays were performed on crude extracts of Ehrlich ascites tumor cells prepared according to the procedure in (6). Protein concentrations as measured by the method of Lowry *et al.* (9) ranged from 3–8 mg/ml of extract.

Measurements of feedback inhibition in

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[2] The abbrevations used are: MTI 6-methylthioinosine, 6-(methylmercapto)-purine ribofuranoside; cMTIMP, 6-methylthioinosine-3′,5′-cyclic monophosphate; AMP, adenosine 5′-monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; MTIMP, 6-methylthioinosine 5′-phosphate; FGAR, formylglycinamide ribonucleotide; ara-A, arabinosyladenine; EAC/s, Ehrlich ascites tumor cells sensitive to MTI; EAC/MTI, Ehrlich ascites tumor cells resistant to MTI; and PRPP, 5-phosphoribosyl-1-pyrophosphate.

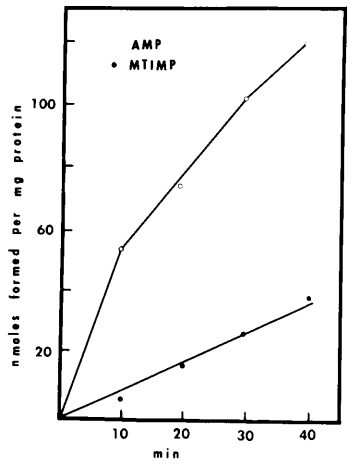

FIG. 1. Cyclic phosphodiesterase activity of resistant tumor extract. Each tube, in duplicate, contained 0.2 ml of tumor cell extract, 7.5 mg protein/ml; 2 μmoles of cAMP, or 2 μmoles of cMTIMP, each in 0.2 ml of buffer; and 1.0 ml of phosphate buffer, pH 7.4. The tubes were incubated for varying lengths of time at 37°C and analyzed as described in (6).

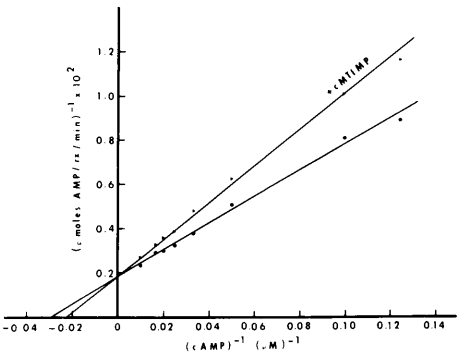

FIG. 2. A Lineweaver–Burk plot of cell free extracts of Ehrlich ascites tumor cells showing inhibition by cMTIMP of cyclic phosphodiesterase when cAMP was used as a substrate. Each tube, in triplicate, contained 0.1 ml tumor cell extract (7.5 mg protein/ml) in a buffer containing 40 m*M* tris-Cl, pH 8.0, 3.75 m*M* 2-mercaptoethanol, 0.1 ml cAMP, concentrations ranging from 100–08 *μm*, 0.1 ml ^{3}H cAMP (85,000 cpm, 23 Ci/m*M*), 5 m*M* $MgCl_2$, and 0.1 ml buffer or 1 m*M* cMTIMP. The final volume was 0.4 ml, and all values given indicate final assay concentrations. The tubes were incubated for 5 min at 30°C and analyzed according to the method of Thompson and Appleman (8).

vitro in whole cells. The measurements of feedback inhibition of *de novo* purine nucleoside synthesis were done by the method of Henderson (10).

Synthesis of 6-^{35}S-methylthioinosine. The method described by Bennett *et al.* (11) was employed to prepare radio- and chemically pure 6-^{35}S-MTI (1 μCi/μmole initial specific activity).

Synthesis of 9-β-D-ribofuranosyl-6-methylthiopurine-3′,5′-cyclic monophosphate (cMTIMP). The chemical synthesis of cMTIMP has been described (11, 12). A pure sample of the cMTIPM was obtained from Dr. R. K. Robins of ICN Research Institute, Irvine, California, for use as a standard.

Results. Celi extracts of the EAC/MTI subline showed cyclic phosphodiestrase activity (Fig. 1) converting cAMP and cMTIMP to 5′AMP and 5′MTIMP, respectively. The cMTIMP was converted to the corresponding 5′-monophosphate much more slowly. At 10 min (Fig. 1), the rate of conversion of cMTIMP to MTIMP was about one-sixth the rate of conversion of cAMP to 5′-AMP. In a preliminary investigation, on crude cell extracts, a Lineweaver–Burk plot (Fig. 2) shows that cMTIMP was a competitive inhibitor of cyclic phosphodiesterase when cAMP was used as a substrate. The apparent K_m for cAMP was $3.1 \times 10^{-5}M$ and the apparent K_i for cMTIMP was $3.0 \times 10^{-3}M$. These figures are approximate as enzyme purification was not performed, and the tumor cells appeared to have multiple cyclic phosphodiesterase activities.

The above results indicate that tumor cells are capable of converting cMTIMP to MTIMP, the pharmacologically active form of the drug. That cMTIMP may be effective as an antitumor agent was demonstrated by the fact that it produced feedback inhibition of PRPP-amidotransferase in both the sensitive and MTI-resistant Ehrlich sublines (Tables I and II). However, the resistant cells showed only 41% inhibition compared to 89% for the MTI-sensitive line for equimolar concentrations ($10^{-3}M$) of cMTIMP. When adenosine ($10^{-3}M$) was used as a control, blockages in both sublines produced about the same degree of inhibition. A similar control using MTI ($10^{-3}M$) gave 67% inhibition in Ehrlich sensitive cells and little or no inhibition in the resistant cells.

TABLE I. FEEDBACK INHIBITION STUDY WITH EHRLICH ASCITES CELLS SENSITIVE TO METHYLTHIOINOSINE[a]

Drugs (1 mM)	FGAR (accumulated CPM)	Inhibition (%)
Control	37,181	0
Adenosine	9,833	74
MTI	12,374	67
cMTIMP	3,759	89

[a] Ten milliliter flasks in triplicate each contained the following in a final volume of 2 ml: 4 μg of azaserine; drugs, 1 μmole/ml; glutamine, 1 μmole/ml; 2-^{14}C-glycine, 0.1 μmole/ml (1 μCi/μmole); glucose, 5.5 μmoles/ml, calcium free Krebs–Ringer phosphate buffer, pH 7.4 and tumor cells, 25 mg/ml wet weight. The tumor cells were preincubated for 5 min with all components except glutamine and 2-^{14}C-glycine, which were then added, and incubation continued for an additional 55 min. Incubations were carried out in air at 37°C in a Dubnoff shaker, and analyzed according to the method of Henderson (10).

TABLE II. FEEDBACK INHIBITION STUDY WITH METHYLTHIOINOSINE RESISTANT EHRLICH ASCITES TUMOR CELLS[a]

Drugs (1 m*M*)	FGAR (accumulated CPM)	Inhibition %
Control	38,320	0
Adenosine	8,563	78
MTI	35,242	8
cMTIMP	22,812	41

[a] The components and procedures were the same as those described in the legend to Table I except that EHR/MTI cells (25 mg/ml wet weight) were used.

Discussion. The 3′,5′-cyclic phosphate derivative of methylthioinosine (cMTIMP) was designed to circumvent tumor cell resistance to MTI because of deficient adenosine kinase activity. A previous report by LePage and Hersh (5) has shown that cMTIMP meets this specification. The data presented here show that one mechanism of action for cMTIMP is feedback inhibition of the *de novo* purine pathway. Presumably, this inhibition occurs only after the conversion of cMTIMP to MTIMP by a cellular cyclic phosphodiesterase—a reaction that occurs in the tumor cells. Studies by Henderson (10) and Henderson and coworkers (4) have shown that the 5′-monophosphate derivative of MTI is required for feedback inhibition of the *de novo* purine pathway. The production of feedback blockade in the MTI-resistant tumor by adenosine but not by MTI also confirms the observation of Lomax and Henderson (3). The competitive inhibition by cMTIMP of a cyclic phosphodiesterase using cAMP as substrate shows that cMTIMP is the 6-methylmercapto-analog of cAMP. This was not altogether unexpected since Bennett *et al.* (11) had demonstrated previously that MTI was the 6-methylmercapto-analog of adenosine.

Summary. The 3′,5′-cyclic phosphate derivative (cMTIMP) of methylthioinosine (MTI) was shown to produce feedback inhibition of the *de novo* purine pathway in an Ehrlich ascites tumor subline resistant to MTI because of lack of adenosine kinase activity for the nucleoside analog.

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