SYNTHESIS AND BIOCHEMICAL EVALUATION OF 6-METHYLTHIOINOSINE-3',5'-CYCLIC MONOPHOSPHATE IN L1210 AND ERHLICH ASCITES CELLS

A Dissertation

Presented to the

Faculty of the Department of Biophysical Sciences

College of Arts and Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

Il-Moo Chang

December 1972

TO

MY FAMILY

ACKNOWLEDGEMENTS

Deepest appreciation is expressed to Dr. A. P. Kimball for his concern, patience and guidance throughout the course of this study. Additional thanks is offered for his timely encouragement and helpfulness during my greatest period of professional growth. The genuine interest and concern of the entire faculty of the Department is also appreciated and will always be remembered. The constructive assistance of my fellow graduate students will also be remembered.

This work was supported by grant CA 12327 from the National Institutes of Health.

오늘이 있기까지 이끌어주고 북돋아준 여러분들과 언제나 머나먼 나를 성장시키기 위해 자신의 보람을 삼은 나의 부모에게 이 조그만 보람을 바친다.

나에게 베풀어진 모든 정신적인 빚은 다음의 젊은 세대에게 돌려지는 것이 모름지기 내가 할 일이라 믿는다.

아울러, 끊임없는 격려와 지도로써 이끌어준 주임 교수Dr. Kimball 에게 감사을 드리면서 . . .

1972년 12월

휴 스 턴

SYNTHESIS AND BIOCHEMICAL EVALUATION OF 6-METHYLTHIOINOSINE-3',5'-CYCLIC MONOPHOSPHATE IN L1210 AND ERHLICH ASCITES CELLS

An Abstract of a Dissertation

Presented to the

Faculty of the Department of Biophysical Sciences

College of Arts and Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

Il-Moo Chang

December 1972

ABSTRACT

Methylthioinosine-3'.5'-monophosphate (c-MTIMP) was synthesized for use in circumventing tumor resistance to methylthioinosine (MTI). c-MTIMP was hydrolyzed to MTIMP by both bovine cyclic phosphodiesterase and extracts of L1210 cells, and showed potent feedback inhibition of de novo purine biosynthesis in Ehrlich and L1210 cells resistant to MTI as well as on both sensitive cell lines.

The data showed that c-MTIMP entered the cell membrane and was hydrolyzed to MTIMP which inhibited the phosphorylpyrophosphate amidotransferase in both the L1210 and the Erhlich ascites cells.

Also MTI was found to inhibit the PRPP amidotransferase of L1210 cells resistant to MTI whereas the enzyme of Ehrlich cells resistant to MTI was not sensitive to MTI. Adenosine also gave feedback inhibition in the MTI resistant cell lines.

Neither MTI or c-MTIMP inhibitions of DNA polymerase, RNA polymerase and AMP pyrophosphorylase were discerned. Concentrations of MTI and c-MTIMP lower than 10^{-3} M showed decreased feedback inhibition, but adenosine gave almost the same degree of feedback inhibition over a range of 10^{-3} M to 10^{-5} M.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

TABLE Page

CHAPTER I

Synthesis of

9-(β-D-Ribofuranosyl)-6-Methylthiopurine-3',5'-Cyclic Phosphate

INTRODUCTION

For some time before the mechanism of nucleic acid synthesis was fully elucidated, one of the first antineoplastic agents, 8-azaguanine was introduced by Kidder, et al.(1). Recognition of the antineoplastic activity shown by analogs of nucleic acid precursors has not only stimulated the design of carcinostatic antimetabolites, but has also contributed to the basic knowledge in understanding the mechanism of nucleic acid biosynthesis.

Although great efforts have been made to discover antineoplastic agents capable of selective toxicity, no such chemotherapeutic agent has as yet appeared. In addition, the capacity of neoplastic cells to become resistant to antineoplastic agents has brought another serious problem to cancer chemotherapy.

A very common drug resistance problem in model systems is caused by a deficiency of the enzyme (eg. pyrophosphorylase or kinase) which converts the drug in vivo to its active form (9, 22, 36). Also several other types of resistances have been observed experimentally:

a) Decreased uptake of a drug (3),

b) Increased degradation of a drug (4),

c) Decreased affinity of the target enzyme (5),

d) Alteration of a metabolic pathway (6).

In some cases no ready biochemical explanation for the mechanism of resistance is apparent.

In order to control the drug resistance, several attempts have been made such as combination of different drugs and/or synthesis of new drugs which can be converted readily to an active form without the aid of a deleted enzyme (12). The present study is aimed at the synthesis of a drug which can circumvent the resistance to 6-methylthioinosine due to a deficiency of adenosine kinase (19, 22, 51, 52).

The potent antineoplastic agent, 6-methylthioinosine was first synthesized by Hampton, et al. (53). It was soon noted that this drug exhibited potent inhibitory effects in 6-mercaptopurine resistant tumor cell lines (45). It has been generally agreed that the conversion of 6-MTI to 6-MTIMP by adenosine kinase is a prerequisite step in order to exhibit pseudo-feedback inhibition to PRPP amidotransferase in the first step of de novo purine synthesis. Thus, 6-MTI resistant tumor lines show markedly decreased adenosine kinase activity (52). In order to control such a case of resistance, it is reasonable to assume that if the active form of the drug, 6-MTIMP, can be introduced into the resistant tumor cells, an inhibitory effect could be achieved. However, there is evidence that intact nucleotides can not penetrate the cell membrane. Therefore,

the structure of 6-MTIMP was modified to give the 3',5'-cyclic phosphoester bond. The structure of c-MTIMP becomes similar to c-AMP, and could be considered an analog of c-AMP. It is known that cyclic AMP is capable of penetrating cell membranes and that it is hydrolyzed to 5'-AMP by a cyclic phosphodiesterase. On the basis of the same reasoning, c-MTIMP should be hydrolyzed to MTIMP by the cyclic phosphodiesterase. With this rationale, c-MTIMP was synthesized and its biochemical effects were evaluated in experimental animal models.

SYNTHESIS

In order to thiate the purine moiety of inosine, the three hydroxyl groups of the ribose moiety were blocked with acetyl groups. Treatment of inosine (I) with acetic anhydride in pyridine yielded the 2',3',5'-tri-O-acetyl-inosine (8). This blocked nucleoside was then thiated with phosphorus pentasulfide in boiling pyridine to give II, 2',3',5'-tri-O-thioinosine, which was then deacetylated with sodium methoxide in anhydrous methanol to give 6-thioinosine, III.

Before the phosphorylation of 5'-OH, 6-thioinosine was treated with perchloric acid and dimethoxypropane in acetone to give the 2',3'-O-isopropylidene-6-thioinosine, IV (9, 10). For the phosphorylation of 5'-OH of IV, the phosphorylating agent, 2-cyanoethyl phosphate was freshly prepared (11). Then compound IV was reacted with 2-cyanoethyl phosphate in the presence of dicyclohexylcarbodiimide in pyridine solution for two days at room temperature (13). The 2',3'-O-isopropylidene group of VI was hydrolized under acidic conditions to yield VII, 6-thioinosine 5'-cyanoethyl phosphate. VII was treated with lithium hydroxide to give VIII, thioinosine 5'-phosphate.

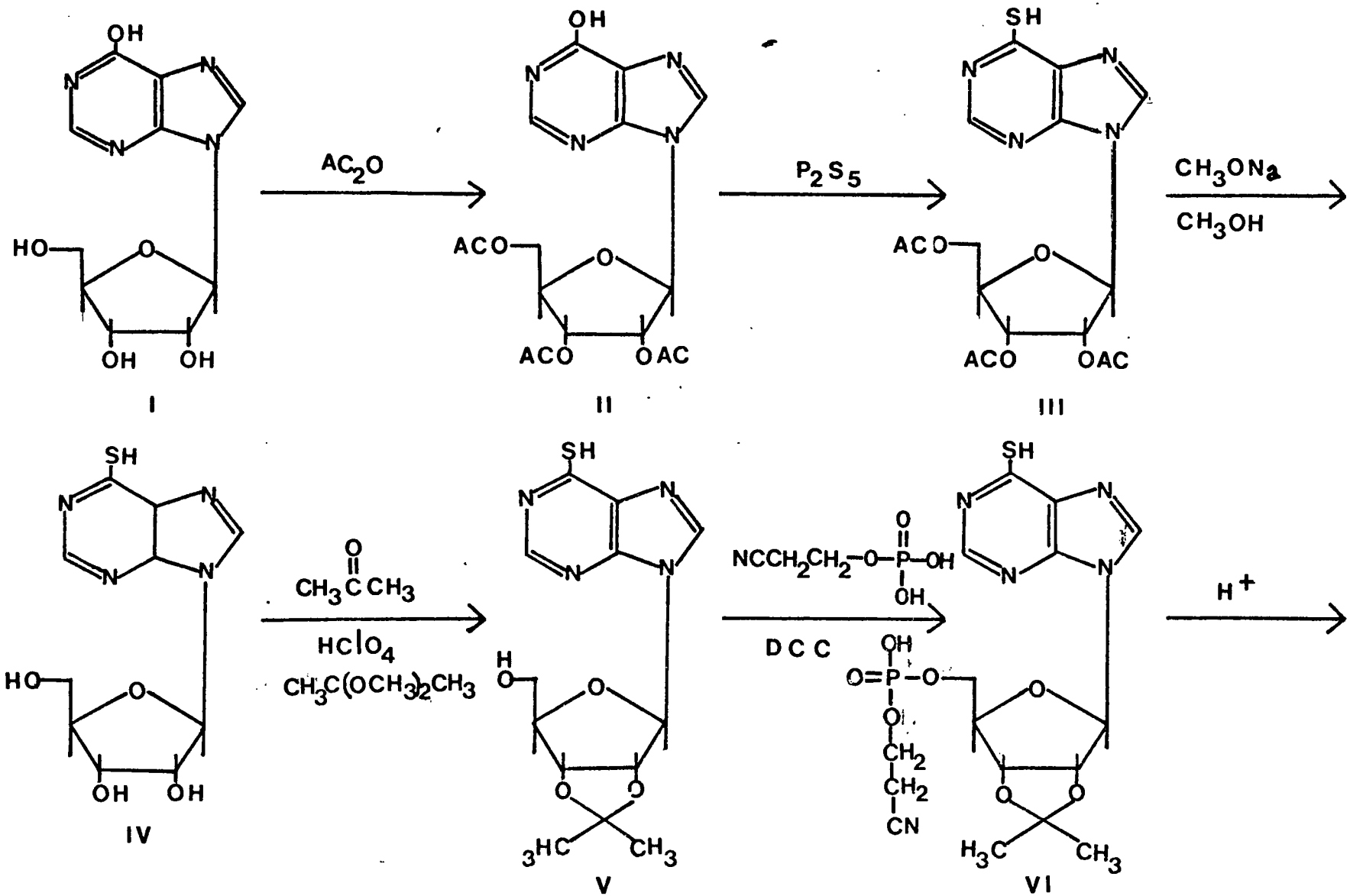
The thiol group of TIMP was methylated with methyl iodide under alkaline conditions to yield IX, 6-methylthioinosine

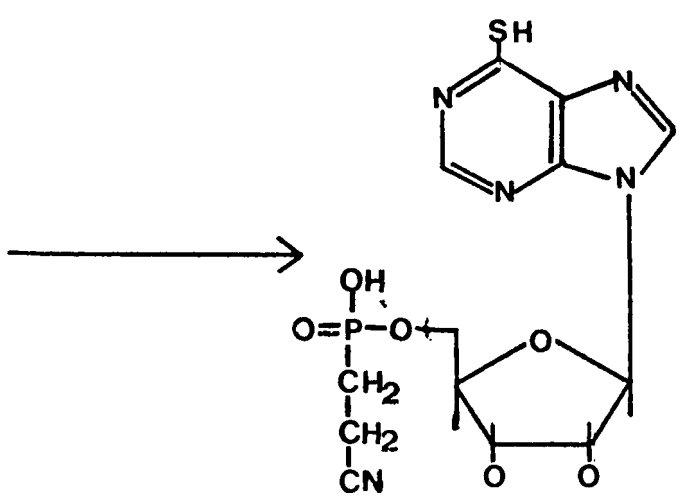
5'-phosphate, which was finally cyclized with morpholine-DCC in pyridine to give X, 6-methylthioinosine-3',5'-cyclic phosphate (13).

The crude product, X, was applied to a DEAE-cellulose column for chromatography in order to obtain pure c-MTIMP.

Figure I

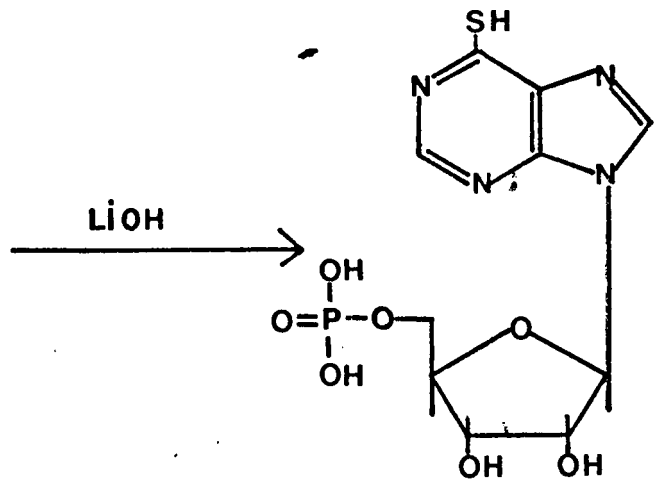
SYNTHETIC PROCEDURE OF c-MTIMP


OH
HO
O
OH OH
I
AC_2O
ACO
OAC
II
P_2S_5
SH
III
CH_3ONa
CH_3OH
IV
$CH_3C(=O)CH_3$
$HClO_4$
$CH_3C(OCH_3)_2CH_3$
V
$NCCH_2CH_2-O-P(=O)(OH)-OH$
DCC
VI
H^+



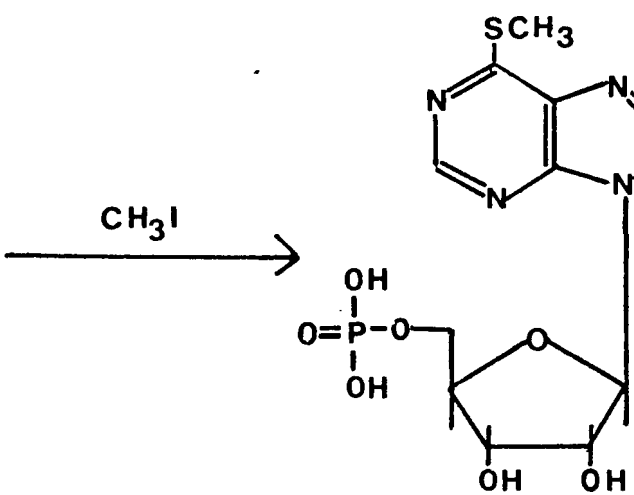
VII

LiOH



VIII

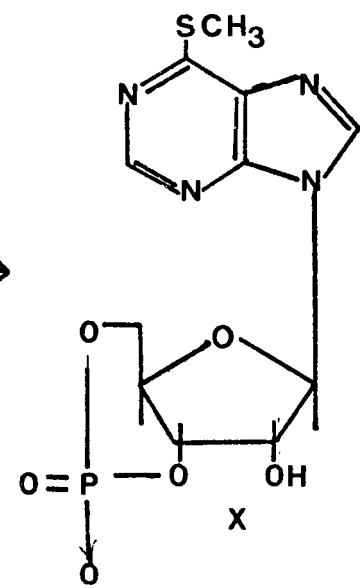
CH_3I



IX

MORPHOLINE

DCC



X

EXPERIMENTAL

9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-9H-hypoxanthine (II)

To a suspension of 50 g (0.19 mole) inosine in 1.5 l of dry pyridine was added 70 ml (0.75 mole) of acetic anhydride. The reaction mixture was stirred at room temperature for 24 hours. The excess acetic anhydride was decomposed with 250 ml of methanol, and, then, the resulting solution was evaporated to dryness *in vacuo* to give crude porduct II. This crude product was recrystallized from water (yield 90%).

2',3',5'-tri-O-acetyl-6-thioinosine (III)

To a solution of 50 g (0.127 mole) of II in 2.5 l of dry pyridine was added 140 g (0.6 mole) of phosphorus pentasulfide. The reaction mixture was heated under reflux for 4.5 hours, while protected from moisture. The resulting dark solution was chilled to room temperature, and it was poured into 4 l of cold water; then this aqueous solution was evaporated to dryness *in vacuo*. The residue was triturated with hot water. and then collect on a filter to give crude product III. Further purification from hot water was done until no frothing occurred. The white solid obtained was dried over phosphorus pentaoxide for two days *in vacuo* (yield 70%).

6-thioinosine (IV)

30 g (0.76 mole) of 2',3',5'-tri-O-acetyl-6-thioinosine was added to 2.5 l of anyhdrous methanol (redistilled with $CuSO_4$) which contained 51.2 g (0.95 mole) of sodium methoxide. The reaction mixture was heated under reflux for 4 hours while protected from moisture. The reaction mixture was cooled to room temperature, and neutralized to pH 7 with acetic acid. The resulting solution was placed under refrigeration overnight, and then the white crystals were collected by filtration. Purification was effected by treating the aqueous suspension of crude product with 3 M ammonium hydroxide to give a clear solution and then neutralization with glacial acetic acid (yield 50%).

2',3'-O-isopropylidene-6-thioinosine (V)

30 ml (0.24 mole) of 2,2-dimethoxypropane and 40 ml (0.64 mole) of 70% perchloric acid were added to a 1.5 l round bottom flask, and the reaction mixture was stirred for 5 minutes at room temperature. Then 20 g (82 mmole) of 6-thioinosine was added quickly and the resulting clear yellow solution was stirred for 20 minutes at room temperature. The reaction was stopped by adding 100 ml of pyridine. A white precipitate formed within 15 seconds. Water (450 ml) was added to the solution followed by 25 ml of 15 N ammonium hydroxide. The resulting

solution was concentrated to about 700 ml *in vacuo* at which time most of the acetone was removed. Twenty-five ml of 15 N ammonium hydroxide was added to the concentrated reaction mixture with continuous stirring. After the solution became clear, it was again concentrated to 450 ml *in vacuo* at 30° C. The resulting solution was extracted with 200 ml of dichloromethane. The aqueous layer was collected and adjusted to pH 6 with acetic acid. White crystals were precipitated immediately. The collected crystals were washed with two 50 ml portions of cold water and dried over phosphorus pentaoxide *in vacuo* for two days (yield 70%).

2',3'-O-isopropylidene-6-thioinosine-5'-cyanoethyl phosphate (VI)

Fourteen and one half g (44.8 mmole) of 2',3'-O-isopropylidene-6-thioinosine was added to 90 mmole (90 ml of stock solution) of 2-cyanoethyl phosphate, followed by 100 ml of dry pyridine (distilled with calcium hydride). The resulting solution was concentrated to an oily form *in vacuo* at 30° C. The oil residue was then taken up in 100 ml of dry pyridine and concentrated. This process was repeated three times in order to remove water in the reaction mixture. Next, 700 ml of dry pyridine containing 64 g (260mmole) of dicyclohexylcarbodiimide was added to the oil in a reaction flask, and the flask was tightly sealed and stirred for 2 days at room temperature. The insoluble precipitate of dicyclohexylurea was

filtered off through a charcoal-Celite pad. The filtrate was evaporated to a gummy residue in vacuo at 35° C. The next step was followed without further purification.

6-thioinosine-5'-cyanoethyl phosphate Ba salt (VII)

The gummy residue obtained was dissolved in 800 ml of water, and the solution was extracted with 400 ml of chloroform. The chloroform layer was discarded and the aqueous layer was acidified with HCl to give a final concentration of 0.3 N HCl. The acidified aqueous solution was stirred for 24 hours at room temperature in order to hydrolyze the isopropylidene group. Then the solution was neutralized with 6 N sodium hydroxide solution and the next step was followed without further purification.

6-thioinosine-5'-phosphate (VIII)

The aqueous solution containing VII was diluted with lithium hydroxide to give a final concentration of 0.5 N lithium hydroxide solution. The solution was heated for 15 minutes at 100° C. A slight precipitate of lithium phosphate which formed was filtered off. Then the filtrate was stirred for 15 minutes with Dowex-50 cation exchange resin. The resin was removed by firtration and the filtrate was carefully adjusted to pH 7.5 with barium hydroxide. The resulting cloudy solution was then

filtered through a charcoal-Celite pad and the filtrate was concentrated to 500 ml (about 1/2 volume) in vacuo at 30° C. This concentrated solution was added to 2 volumes of absolute ethanol. Immediately a white precipitate formed. The white precipitate was collected by centrifugation (5000 rpm, Beckman J-21) at 3° C. The yield was 20%.

6-methylthioinosine-5'-phosphate Ba salt (IX)

An aqueous solution of VIII Ba salt (5 g, 100 mmole) was stirred for 15 minutes with Dowex-50 cation exchange resin in order to remove barium and the resin was removed by filtration. Sulfuric acid (0.1 N) was added dropwise to the filtrate to ensure the removal of residual barium. After the $BaSO_4$ was filtered off, the free acid form of 6-MTIMP was converted to its di-sodium salt form with sodium hydroxide solution. To 4 g (9mmole) of the disodium salt of 6-MTIMP in 27 ml of water and 9 ml of 1 N sodium hydroxide solution was added 0.75 ml of methyl iodide. The reaction mixture was stirred vigorously for 24 hours at room temperature. The resulting reaction mixture was evaporated to dryness in vacuo at 35° C. Then, the residue was dissolved in 50 ml of water and acidified by Dowex-50 cation exchange resin. After the resin was filtered off, the filtrate was adjusted to pH 8 with barium hydroxide solution. The resulting cloudy solution was filtered through a charcoal-Celite pad, and the filtrate was added to 2 volumes

of absolute ethanol to give a white precipitate. This precipitate of the Ba salt of 6-MTIMP was collected by centrifugation at 3° C (5000 rpm). The yield was 30%.

6-methylthioinosine-3',5'-cyclic phosphate (X)

Two and twentyseven hundredth g (6 mmole) of the free acid form of 6-MTIMP and 1.8 g (6 mmole) of 4-morpholine-N,N'-dicyclohexylcarboxamidine were dissolved in 150 ml of pyridine containing 20 ml of water. This solution was evaporated to dryness in vacuo at 35° C and the residue was evaporated in the presence of 50 ml of pyridine to dryness in vacuo at 35° C. This process was repeated three times in order to remove residual water. Then, the 4-morpholine-N,N'-dicyclohexylcarboxamidine salt of 6-MTIMP was taken up in 600 ml of dry pyridine, and added dropwise for 2 hours via the reflux condenser to a boiling solution of DCC (4.19 g, 20 mmole) in 600 ml of pyridine while the system was protected from moisture. Reflux was continued for an additional 3 hours and the reaction mixture was evaporated to dryness in vacuo at 35° C. The residue was dissolved in 300 ml of water and left for 2 hours at room temperature until dicyclohexylurea precipitated out. The insoluble dicyclohexylurea was filtered off through a Celite pad. The clear aqueous filtrate was placed in a refrigerator of 4° C for 4 hours. Residual dicyclohexylurea was filtered off and the filtrate was concentrated to a small volume (about 2 ml) in vacuo at 30° C.

Then this crude reaction product containing c-MTIMP was applied to a DEAE-cellulose (bicarbonate form) column chromatography in order to purify c-MTIMP.

Separation of c-MTIMP by DEAE-cellulose (bicarbonate form) Column Chromatography

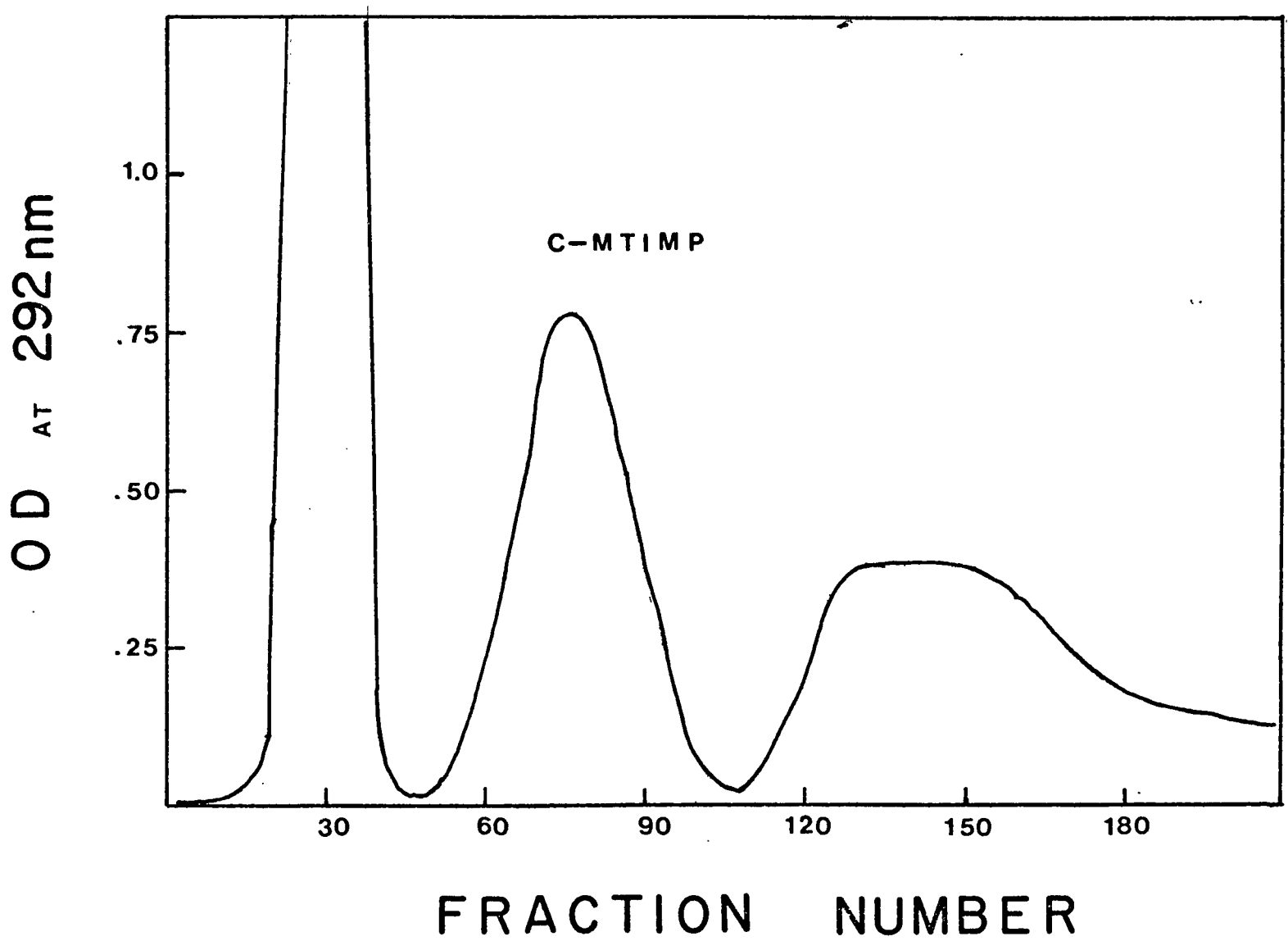
Two ml of the concentrated crude reaction product was dissolved in 3 ml of 0.001 N triethylammonium bicarbonate, and this solution was carefully poured in at the top of a DEAE-cellulose (HCO_3^-) column (50 cm x 3 cm) which was pre-equilibrated with 0.001 N triethylammonium bicarbonate. Elution was carried out by using a linear salt gradient with 0.001 M triethylammonium bicarbonate (total, 3 l), pH 7.5, in the mixing chamber and 0.1 M triethylammonium bicarbonate (total, 3l) in the reservoir. Each fraction of about 8 ml was collected and measured spectrometrically at 292 mμ. c-MTIMP appeared in fractions 50 through 100. The combined fractions were evaporated to dryness *in vacuo* at 30° C. The residue was dissolved in water and stirred with Dowex-50 (Na^+ form) to converted the c-MTIMP to its sodium form. Afterwards, the resin was filtered off and the filtrate was evaporated to dryness *in vacuo* at 30°C. A mixture of acetone and ethanol (1:1) was added to the residue and the mixture was evaporated to dryness *in vacuo* at 30° C. This process was repeated several times. A final yeild of 130 mg of pure compound was obtained.

Figure 2

ELUTION PROFILE

OF

CRUDE PRODUCTS ON DEAE-CELLULOSE (HCO_3^-) COLUMN


OD AT 292nm
1.0
.75
.50
.25
C-MTIMP
30
60
90
120
150
180
FRACTION NUMBER

Identification of c-MTIMP

The c-MTIMP was characterized by ultraviolet spectroscopy, thin layer chromatography, paper chromatography and by enzymatic hydrolysis to MTIMP. Due to the methylation of the 6-thiol group in the purine moiety, a maximum absorption peak at 292 mu at pH 7 was observed. Purine, nucleoside, nucleotide, and cyclic nucleotide were separated by PEI-cellulose thin layer plate and paper (Whatman # 1) chromatography, in various solvent systems. A bright yellow spot due to the 6-methylthio group in the purine moiety was observed under a short wave UV lamp, whereas a blue spot was observed due to the 6-thiol group on the purine on both TLC plates and papers. Rf values of the intermediates and final products are described on Table 2.

In order to characterize the 3',5'-cyclic phosphoester bond of c-MTIMP, the final product was reacted with bovine cyclic phophodiesterase. Cyclic AMP was also reacted with the enzyme as the control. After the enzyme reaction was terminated, the reaction mixture was spotted on a PEI-cellulose plate and the TLC plate was developed in aqueous solution of 0.3 M LiCl, 5 mM Tris-HCl (pH 8.4). The 5'-MTIMP derived from c-MTIMP by the enzyme reaction was observed. As Table 1 shows, c-MTIMP and c-AMP were substrates for the cyclic phosphodiesterase which hydrolyzes specifically the 3',5'-cyclic phosphoester bond.

Table 1

Hydrolysis of c-MTIMP and c-AMP by

Bovine Cyclic Phosphodiesterase

Time (Min)	Relative amount of			
	c-AMP	5'-AMP	c-MTIMP	5'-MTIMP
0	++++	----	++++	----
15	+++	+	+++	+
30	+	+++	++	++

Each reaction tube contained 0.5 μmoles of substrate and 0.04 units of beef heart cyclic 3',5'-phosphodiesterase dissolved in Krebs-Ringer phosphate buffer (no $CaCl_2$) in a final volume of 0.1 ml. The reaction tubes were incubated at 37° C, and spotted on PEI-cellulose plates and developed in 0.3 M LiCl and 5 mmole Tris-HCl, pH 8.4.

Solvent System	Time	Type
1) Isopropyl alcohol:H_2O:NH_4OH (7:2:1)	12 hrs.	Descending (Whatman # 1)
2) n-BuOH:H_2O:HAc (5:3:2)	5 hrs.	Descending (Whatman # 1)
3) 0.3 M LiCl in 5 mmole Tris-HCl, pH 8.4	15 Cm.	TLC (PEI-cellulose)
4) 0.1 N Na_2HPO_4	3.5 hrs.	Descending (Whatman # 1)

Table 2

Rf Value of Some Analogs of Purine, Nucleoside, and Nucleotide

Analogs	1	2	3	4
Adenine	0.55	-	-	-
Inosine	0.50	0.40	-	-
6-Mercaptopurine	0.54	-	0.38	-
6-Methylmercaptopurine	0.70	-	0.48	-
2',3',5'-tri-O-Ac-Inosine	-	0.76	-	-
2',3',5'-tri-O-Ac-Thioinosine	0.69	-	-	-
6-Thioinosine	0.53	0.73	0.51	(1.10)
2',3',5'-O-Isopropylidene-6-Thioinosine	0.72	(1.23)	-	(0.01)
6-Methylthioinosine	0.79	-	0.61	-
6-Thioinosine-5'-phosphate	0.18	0.30	0.10	(1.60)
6-MTIMP	0.22	-	0.08	-
AMP	-	0.22	0.10	-
c-MTIMP	0.67	-	0.43	-

() = relative to Adenine

MATERIALS AND INSTRUMENTS

Several reagents that were not commercially available were synthesized.

2-cyanoethyl phosphate, Ba salt, phosphorylating agent

Eighteen ml (0.2 mole) of phosphorus oxychloride was mixed with 200 ml of anhydrous ether in a three-neck flask fitted with a thermometer. The reaction flask was placed in an ice-salt bath, and a mixture of 16 ml of anhydrous pyridine and 14.2 g (0.2 mole) of hydracrylonitrile was added dropwise to the reaction flask with vigorous stirring. The addition took about 40 minutes in order to keep the reaction temperature at -10° C. A white solid precipitated out, but was not removed. The resulting reaction solution, together with the white solid, was poured into 750 ml of water, 80 ml of pyridine and 300 g of ice. To this resulting solution was added a solution of 100 g of barium acetate in 300 ml of water. Then, the solution was placed in the refrigerator at 4° C for 5 hours to allow aggregation of the barium phosphate. The barium phosphate was removed by filtration through a Celite pad, and the filtrate was concentrated to about 800 ml *in vacuo*. At this step, most of the ether was removed. To the concentrated solution was

added 1600 ml (2 volumes) of absolute ethanol to precipitate out the barium salt of 2-cyanoethyl phosphate. The product was collected by filtration.

The product was identified by its infrared spectrum determined in a KBr pellet [peaks at 3200 (broad), 2200, 1620, and 1380 (short)].

A standard stock solution of the free acid form of 2-cyanoethyl phosphate containing 1 mmole/ml was prepared. The Ba salt (16.1 g) of 2-cyanoethyl phosphate was dissolved in 150 ml of water, and stirred with Dowex-50 cation exchange resin for 15 minutes and the resin was filtered off. To a filtrate was added 1 N H_2SO_4 dropwise until $BaSO_4$ no longer precipitated out. After filtration of residual $BaSO_4$, the filtrate was mixed with 100 ml of pyridine, and evaporated to dryness in vacuo at 35° C. This process was repeated several times until residual water was removed. The gummy residue was dissolved in dry pyridine to give a final volume of 50 ml.

4-morpholine-N,N'-dicyclohexylcarboxamidine

DCC, 4.1 g (20 mmole), and 4 ml (40 mmole) of morpholine were dissolved in 10 ml of t-butyl alcohol. The solution was heated under reflux for 4 hours, then cooled and left overnight at room temperature. The resulting white crystals were washed first with a small amount of t-butyl alcohol and then with petroleum ether (yield 80%).

Preparation of DEAE-cellulose (bicarbonate form) column

Forty g of DEAE-cellulose were suspended in 3 l of distilled water and the fine particles were removed by decantation. This process was repeated three times. The washed exchanger was stirred with 5 bed volumes of 0.1 N NaOH solution overnight, and collected in a sintered glass funnel. The exchanger was washed thoroughly with distilled water and suspended in 2 volumes of 2 M ammonium bicarbonate solution.

The resin suspension in ammonium bicarbonate was stirred for one day and then packed in a 50 cm x 3 cm column. The column was washed with 2 l of 2 M ammonium bicarbonate solution to ensure that the resin was in the bicarbonate form. The exchanger was then thoroughly washed with distilled water before use.

Triethylammonium bicarbonate

Small pieces of dry ice (to supply CO_2) were dropped into 138 ml (1 mole) of triethylamine in an ice-water bath until the pH of the solution reached 7.5. It was important to check the pH of this buffer just prior to use.

Beef heart phosphodiesterase-3',5'-cyclic nucleotides, Sigma Co.

Inosine, Sigma Co.

Infrared spectroscope, Perkin-Elmer 700

Beckman DB-G spectrophotometer, Beckman Instruments Inc.

Fraction collector, LKB 7000 Ultro Rac. LKB-Produkter, AB.

Beckman J-21 centrifuge, Beckman Instruments Inc.

Chapter 2

Evaluation of Some Biochemical Effects of c-MTIMP in L1210 and Erhlich Ascites Tumor Cells

INTRODUCTION

PRPP amidotransferase (E. C. 2.4.2.14), the first enzyme of de novo purine biosynthesis, catalyzes the reaction in which 5'-phosphoribosylpyrophosphate, glutamine, and water interact to form 5'-phosphoribosyl-1-amine, glutamate and pyrophosphate. Phosphoribosylamine enters a sequence of reactions that eventually yields IMP.

Wyngaarden, et al. (20) found that this enzyme was susceptible to end-product inhibition, and according to binding studies of inhibitors, this enzyme has more than one binding site for compounds that are analogs of 6-hydroxy- and 6-aminopurine ribonucleotides. The binding site for 6-hydroxypurine ribonucleotide analogs was found to be separate from the binding

---

Abbreviations used are: PRPP, 5'-phosphoribosyl pyrophosphate; IMP, inosine-5'-monophosphate; AMP, adenosine-5'-monophosphate; GMP, guanosine-5'-monophosphate; ATP, adenosine-5'-triphosphate; 6-TIMP, 6-thioinosine-5'-monophosphate; MTIMP, 6-methylthioinosine-5'-monophosphate; 6-MP, 6-mercaptopurine; MTI, 6-methylthioinosine; c-TIMP, 6-thioinosine-3',5'-phosphate; c-MTIMP, 6-methylthioinosine-3',5'-phosphate; FGAR, Formyl glycine amide ribotide; L1210/MTI, L1210 cells resistant to MTI; EAC/MTI, Ehrlich ascites cells resistant to MTI.

site for 6-aminopurine ribonucleotide analogs, and, in addition, another distinct binding site for other purine nucleotides such as ATP may be located on the enzyme. Furthermore, binding of two different inhibitors, such as AMP plus GMP, provide cooperative inhibition (21). However, it is uncertain whether or not TIMP and MTIMP bind to the same site (22). In addition, Paterson, et al. (44) suggested that the two drugs may bind to the same site, presumably the one for GMP, on the basis of in vivo studies.

It is generally agreed that the conversion of MTI and 6-MP to their corresponding ribonucleotides is a prerequisite step to exhibit feedback inhibition of PRPP amidotransferase (19, 33, 45, 47, 51).

Tumor cell lines resistant to 6-MP or MTI oftentime show marked deficiencies of phosphorylating enzyme activity: GMP-IMP pyrophosphorylase or adenosine kinase, respectively, In contrast to the above, Henderson, et al. (46) observed that 6-benzylthioguanine inhibited PRPP amidotransferase without phosphorylation of its intact form.

Montgomery, et al. (14) synthesized c-TIMP in order to circumvent 6-MP resistance due to loss of GMP-IMP pyrophosphorylase, and found c-TIMP was not effective against HEp-2 cells resistant to 6-MP. Unlike their observations, recently LePage, et al. (48) observed that c-TIMP inhibited growth of L1210 cells resistant to 6-MP.

While the present studies were being carried out, Meyer, et al. (43) reported on the synthsis of c-MTIMP, and LePage, et al. found that this compound inhibited the growth of Erhlich ascites cells resistant to MTI.

MATERIALS AND METHODS

ANIMALS, CELLS AND ENZYMES:

L1210/MTI cells were a gift from Dr. W. R. Laster, Jr. of Southern Research Institute at Birmingham, Alabama. Female BDF_1 mice (Jackson Memorial Laboratories, Bar Harbor, Maine) were implanted intraperitoneally with 10^6 L1210/MTI cells and injected intraperitoneally once daily for six consecutive days with 10 mg/kg MTI in physiological saline, beginning 24 hours after implantation. The cells were then harvested and transplanted on the seventh day after implantation and were drug treated as previously described.

EAC/S and EAC/MTI cells were obtained from Dr. A. P. Kimball. Female Swiss albino mice (Jackson Memorial Laboratories) were implanted with 2×10^6 Erhlich ascites cells resistant to MTI. Drug suppressions with 10 mg/kg MTI in physiological saline were started 24 hours after implantation and continued once daily for three consecutive days.

For *in vitro* whole cell experiments tumor cells were removed by capillary pipettes after incision of the peritoneal cavity and the ascitic fluid was removed by centrifugation at approximately 1490 g for two minutes in an IEC clinical centrifuge. The yield of packed cells in the pellet was approximately

1.4×10^9 cells/ml. The packed cells were resuspended in 5 volumes of ice-cold double distilled water for 30 seconds to lyse the red blood cells; then 5 volumes of 1.8 % NaCl solution was added and thoroughly mixed. This suspension was centrifuged as before. The cells were washed once in 0.9 % saline and dilute 1:8 with calcium-free Krebs-Ringer phosphate medium (23) for the feedback studies or diluted 1:4 with Fischer medium (8) without serum (Hyland Laboratories) for the measurement of DNA and RNA synthesis.

For the enzyme experiments, broken cell preparations and cell-free extracts were used. For the former, the cells, after proper washing were suspended in 4 volumes of ice-cold double distilled water for 10 minutes in the cold and disrupted with 20 strokes using a glass tissue homogenizer. This broken cell preparation was used for enzyme assays without further treatment.

Cell-free extracts were prepared as follows: the cells were suspended in 10 volumes of ice-cold double distilled water in the cold and homogenized with 20 strokes in a glass tissue homogenizer and the cell debris was centrifuged down in a Beckman J-21 centrifuge at 11,000 rpm for 90 minutes. The supernatant fluid was saved for subsequent enzyme assays. One ml of extract contained approximately 3 mg of protein. Protein content was measured by the methods of Lowry, et al. (29).

SURVIVAL STUDIES:

Female BDF_1 mice differing in weight by ± one gram were used. The average weight of approximately 20 g/mouse was used and the mice were divided into groups of ten; each mouse was implanted with 10^6 L1210/MTI cells. MTI (10 mg/kg) or saline in the amount of 0.2 ml was injected intraperitoneally once daily for 6 consecutive days starting 24 hours after implantation. The day of death was recorded and the average life span was subsequently calculated.

MEASUREMENT OF FEEDBACK INHIBITION:

Henderson's modification (26) of LePage and Jones' procedure (25) was employed to measure the degree of feedback inhibition of PRPP amidotransferase.

In the presence of azaserine, which irreversibly inhibits FGAR amidotransferase (28), FGAR accumulates. The amount of FGAR accumulation is a measure of the PRPP amidotransferase inhibition assay. A decrease in FGAR formation in the presence of exogenous purines or MTI can be ascribed to feedback inhibition. Cells (0.2ml - 1:8 dilution) were suspended in calcium-free Krebs-Ringer phosphate buffer containing 5.5 mM glucose. Azaserine (2 ug/ml - 6.0×10^{-4} M) and drugs (1 mM) or medium, as control, were added. Reaction flasks were pre-incubated for 5 minutes at 37° C with shaking in air in a Precision

Scientific Dubnoff Metabolic Incubator. Following this, glutamine (1 mM) and glycine-2-^{14}C (1 μmole, specific activity, .1 mCi/mM) were added to the reaction flask making a final volume of 2 ml and incubated for an additional 55 minutes. The reaction was stopped with 0.5 ml of ice-cold 2.6 M perchloric acid in the cold. Cell pellets were collected by centrifugation and 0.5 ml of 2.6 M KOH was added. After the insoluble $KClO_4$ was removed by centrifugation, the supernatant was poured on top of a Dowex-1-X-8-formate column (10 mm x 35 mm). After adsorption, the columns were eluted with 30 ml of formic acid; the effluent was discarded and the columns were re-eluted with 15 ml of 4 M formic acid. One ml of effluent was mixed with 10 ml Aquasol (New England Nuclear) and the radioactivity was measured in a Packard Liquid Scintillation Spectrometer, Model 3380.

MEASUREMENT OF INCORPORATION OF GLYCINE-2-^{14}C INTO ACID-INSOLUBLE POLYNUCLEOTIDES IN VIVO:

BDF_1 mice bearing 6-day implants of L1210 cells each received MTI (82 mg/kg) or c-MTIMP (100 mg/kg) in saline or saline alone, as control. One hour later, glycine-2-^{14}C (2 μmoles - specific activity, 1 uCi/umole) was injected intraperitoneally and metabolic utilization was allowed to proceed for one hour. The mice were sacrificed and the ascites cells were harvested. The cells were extracted four times with 10 volume portions of

ice-cold 5 % trichloroacetic acid (TCA). After these washes were removed by centrifugation, residual TCA was extracted two times with 10 volume portions of ice-cold 95 % ethanol. Three volume portions of 5 % TCA was added to the precipitates and boiled at 100° C for 15 minutes to hydrolyze polynucleotides. Thereafter, the cell debris was removed by centrifugation and 1.0 ml of supernatant was subsequently dissolved in 10 ml of Aquasol and counted.

MEASUREMENT OF DNA AND RNA SYNTHESIS IN VITRO:

Cells were suspended in Fischer medium (3.7×10^7 L1210 cells; 1:4 dilution). One mM of drugs and cells were incubated for 25 minutes at 37° C. Tritiated-TdR(Methyl) (3 µmoles - specific activity, 5 µCi/umole) or tritiated UR (2.0 umoles - specific activity, 5 µCi/umole) was added to the reaction flask. At the indicated time, 2 ml of samples were delivered into chilled tubes containing equal volumes of cold 10 % cold TCA. The precipitates were extracted four times with 4 ml portions of 5 % ice-cold TCA. Then residual acid was extracted two times with 4 ml portions of ice-cold 95 % ethanol. The precipitates were solubilized in 1 ml of Protosol (New England Nuclear) for 2 hours at 55° C. The samples were counted in 10 ml of toluene containing 6 g/l PPO, and 0.75 g/l POPOP.

RNA POLYMERASE ASSAY:

The assay method was previously described (18). Each reaction tube contained:

(1) 100 µg of native DNA in 50 umoles Tris-HCl buffer, pH 7.9,
(2) 300 µl of broken cells (1:4 dilution),
(3) 25 µl of NaF (20 µmoles),
(4) 25 µl of $MgCl_2$ (2 µmoles),
(5) 25 µl of $MnCl_2$ (2 µmoles),
(6) 500 µl of drug or H_2O solution,
(7) 100 µl of ATP, CTP, GTP (250 µmoles of each),
and (8) ^3H-UTP (250 µmoles, 25 µCi/µmole),
to give a final volume of 1.025 ml.

These reaction tubes minus the substrates were preincubated for 30 minutes. The tubes were then placed in an ice bath and the substrates were added. Incubation, as before, was continued an additional 20 minutes. The reaction was stopped with 100 µl of 50 % TCA in the cold. The precipitates were washed, solubilized in Protosol and counted in a scintillation counter as has been previously described.

DNA POLYMERASE ASSAY:

The assay was a modification of a method previously described (30, 31). Each reaction tube consisted of:

(1) 50 µl of 1 M Tris-HCl, pH 8.0 with 0.1 M $MgCl_2$,

(2) 100 µl of 750 µg/ml creatine phosphokinase, 50 mM creatine phosphate and 20 mM ATP,

(3) 100 µl of 0.1 mM dATP; 0.1 mM dGTP; 0.1 mM dCTP and 0.1 mM TTP-^3H

(4) 50 µl of 2mg/ml denatured calf thymus DNA,

(5) 150 ul of broken cell preparation (1:4 dilution)

and (6) drug or water

to give a total volume of 0.5 ml.

The reaction tubes minus the substrates were pre-incubated for 15 minutes (sometimes 30 minutes) at 37° C. The tubes were then placed in an ice bath and the substrates were immediately added and additional incubation was continued for 15 minutes. The reaction was stopped with 50 µl of 50 % TCA in the cold. The precipitates were washed and solubilized and the radioactivities incorporated into DNA were counted as previously described.

ADENOSINE KINASE ASSAY:

The enzyme activity was measured in whole cell suspensions and cell-free extracts (32, 52). MTI-^{35}S was prepared by Dr. A. P. Kimball using the method of Moravek, et al. (33). Reaction mixture were composed of 200 ul of 1 mM MTI-^{35}S, 0.2 ml whole cell suspension in a total volume of 2.0 ml of calcium-free Krebs-Ringer phosphate buffer with 5.5 mM glucose. The

tubes were incubated for 1 hour at 37° C and the reaction was stopped by the addition of 500 µl of 2.6 N $HClO_4$ and neutralized with 0.5 ml of 2.6 N KOH. After $KClO_4$ was removed by centrifugation, aliquots of the supernatants and authentic cold compounds were co-chromatographed on Whatman # 3 paper with 5 % Na_2HPO_4 as the descending migrating solvent for a distance of 30 cm. After the chromatograms were dried, ultraviolet absorption spots were cut off and counted in a tolune fiour.

For the enzyme assay in cell-free extract, the reaction tubes were composed of:

(1) 100 µl of 10mM MTI-^{35}S,

(2) 100 µl of ATP-regenerating system:

(a) 0.75 mg creatine kinase,

(b) 50 mM creatine phosphate,

(c) 10 mM ATP,

(3) 150 µl of cell-free extract (1:4 dilution),

and (4) 150 µl of calcium-free Krebs-Ringer solution made in 5.5 mM glucose.

Tubes were incubated at 37° C for a specified time. The reaction tubes were immersed in boiling water for 3 minutes. The denatured protein was removed by centrifugation, and aliquots of the supernatants were spotted on Whatman # 3 paper and developed and counted as previously described.

AMP PYROPHOSPHORYLASE ASSAY:

The assay method was previously described (36, 40, 41). The reaction mixture contained;

(1) 100 µl of PRPP (1 µmole),

(2) 100 µl of Tris buffer, pH 7.6 (50 umoles),

(3) 200 µl cell-free extract (1:10 dilution - about 600 µg of protein),

(4) 100 µl of adenine-8-^{14}C (0.25 umoles),

and (5) 400 µl of drug solution or water.

The reaction tubes were incubated at 37° C. At the specified time, the reaction was quenched by immersion in boiling water for 3 minutes. After denatured protein was removed by centrifugation, 100 µl aliquots of supernatant were spotted with authentic AMP on PEI-cellulose TLC sheets, developed with 0.2 N formic acid and were air-dried. Then the sheets were developed in the other direction by turning 90° with 0.1 N LiCl as the second migrating solvent. The UV absorption spots were removed and counted in toluene flour.

RESULTS

IN VITRO FEEDBACK STUDIES OF c-MTIMP:

The rationale has been proposed that if c-MTIMP enters the cell menbrane and is hydrolyzed to MTIMP, it should show an appreciable feedback inhibition in both EAC/S and EAC/MTI cells. The amount of FGAR accumulation in azaserine-treated ascites cells was used as the measurement of feedback inhibition of PRPP amidotransferase(25). In order to obtain relative inhibitory effects, adenosine and MTI, which are known potent inhibitors of PRPP amidotransferase, were used.

The data, shown in Table 3, indicate that EAS cells are sensitive to c-MTIMP as well as adenosine and MTI. In addition, EAS/MTI cells which lack adenosine kinase are significantly inhibited by c-MTIMP and adenosine, whereas, MTI does not gave appreciable feedback inhibition as might be expected.

This suggests that c-MTIMP may enter the cell membrane and may be hydrolyzed to MTIMP, which can inhibit PRPP amidotransferase in both EAC/S and EAC/MTI cells. This interpretation seems to reasonable with regards to the observatins of LePage and Hersh(48), that c-MTIMP exhibits growth inhibitory effects against EAC/TGRII cells resistant to MTI.

It should be noted that adenosine still shows significant feedback inhibition against EAC/MTI cells even though this cell line shows resistance to MTI due to a deficiency of adenosine kinase. This could be interpretated to mean that adenosine kinase is unimpaired for the phosphorylation of adenosine but not for the phosphorylation of MTI. It is possible that this enzyme perhaps mutated due to continuous suppression by MTI treatment.

In order to support this explanation, it is cogent to refer to the similar observations of Lomax and Henderson(50) in which adenosine kinase in EAC-R_2 cells resistant to MTI, still phosphorylated adenosine to AMP, but did not phosphorylate MTI to MTIMP. They were able to measure intact adenosine kinase activity while high adenosine deaminase activity was being inhibited by coformycin.

In accordance with their interpretation it was concluded that either adenosine kinase was mutated or two different nucleoside kinases catalyzed the phosphorylation of adenosine and MTI. However, the data obtained from competition experiment with adenosine and MTI have indicated that the two compounds are substrates for adenosine kinase. Therefore, it seems resonable that adenosine kinase may be mutated rather than two different nucleoside kinase exist.

Since theophylline is an inhibitor of at least one of

TABLE 3

FEEDBACK STUDIES IN EAC/S CELLS

Drugs (10^{-3} M)	FGAR Accumulated (cpm)	Inhibition %
Control	37,181	0.0
Adenosine	9,833	74.0
MTI	12,374	67.0
c-MTIMP	3,759	89.0

Each value is an average of two separate determinations.

TABLE 4

FEEDBACK STUDIES IN

ERHLICH ASCITES CELLS RESISTANT TO 6-MTI

Drug (10^{-3} M)	FGAR Accumulated (cpm)	Inhibition %
Control	38,320	0.0
Adenosine	8,563	78.0
MTI	35,242	8.0
C-MTIMP	22,812	41.0

TABLE 5

EFFECTS OF THEOPHYLLINE ON THE FEEDBACK INHIBITION OF EAC/MTI

Drug (10^{-3} M)	FGAR Accumulated (cpm)	Inhibition %
Control	29,900	0.0
Theophylline	25,700	15.0
c-MTIMP	22,100	30.0
c-MTIMP + Theophylline	20,520	31.0
MTI	26,000	13.0
MTI + Theophylline	24,700	18.0

Reaction tubes were prepared as shown in Materials and Methods.

the cyclic phosphodiesterases(37,38), and if c-MTIMP itself does not inhibit PRPP amidotransferase, treatment of the tumor cells with theophylline should relieve the inhibition of PRPP amidotransferase to some extent. The results of such an experiment are shown in Table 5. Under the experimental conditions, 1 mM of theophylline itself inhibits the PRPP amidotransferase to some extent. In the presence of 1 mM theophylline and 1 mM c-MTIMP, the total inhibitory effect appears to be less than sum of the individual inhibitory effects of the two inhibitors at the same concentration. This result suggests that theophylline inhibited the cyclic phosphodiesterase activity; therefore, the formation of MTIMP was impeded. In this regard, c-MTIMP itself may not inhibit PRPP amidotransferase unless it is hydrolyzed to MTIMP, as it was expected under the proposed rationale.

In order to study the feedback inhibitory effects in L1210/S cells by c-MTIMP, The cells were treated with 1 mM adenosine or 1 mM c-MTIMP or 1 mM MTI under identical experimental conditions as employed in the Erhlich ascites cell system. The data, shown in Table 6, show that adenosine, c-MTIMP, and MTI at 1 mM concentrations, significantly inhibit the PRPP amidotransferase of L1210/S cells. Therefore, the feedback inhibition by c-MTIMP seems to work in the same manner as was observed in EAC/S cells.

TABLE 6

FEEDBACK STUDIES IN L1210/S

Drug (10^{-3} M)	FGAR Accumulated (cpm)	Inhibition %
Control	81,212	0.0
Adenosine	32,482	60.0
MTI	31,120	62.0
c-MTIMP	35,450	57.0

TABLE 7

FEEDBACK STUDIES IN L1210/MTI

Drug (10^{-3} M)	FGAR Accumulated (cpm)	Inhibition %
Control	68,580	0.0
Adenosine	31,590	54.0
MTI	30,450	56.0
c-MTIMP	32,565	53.0

However, in L1210/MTI cells, the feedback inhibitory effects of these three drugs appeared somewhat different to those in the EAC/MTI cells. Not only did adenosine and c-MTIMP give rise to significant feedback inhibition, but MTI also exhibited inhibition in L1210/MTI cells as much as in L1210/S cells, although the adenosine kinase activity is deficient in L1210 cells resistant to MTI. Bennett, et al.(36) suggested that MTI resistance in L1210 cells resulted primarily from a deficiency of adenosine kinase. Consequently, no MTIMP could formed in the L1210/MTI cells and PRPP amidotransferase would not be inhibited in L1210/MTI cells.
However, such suggestions do not rule out the possibility that MTIMP alone inhibits the PRPP amidotransferase or MTI itself can be the inhibitor of this enzyme. Furthermore, it has not been reported that MTI does not inhibit the PRPP amidotransferase of L1210 cells.

IN VIVO EFFECTS OF c-MTIMP ON INCORPORATION OF GLYCINE-2-^{14}C INTO POLYNUCLEOTIDES IN L1210 CELLS

Female BDF_1 mice bearing six day implants of the L1210 cells each received 82 mg/kg of MTI, and 100 mg/kg of MTIMP. One hour later, the mice each received glycine-2-^{14}C and metabolic utilization was allowed to proceed for one hour. The incorporation of glycine-2-^{14}C through the de novo purine

TABLE 8

IN VIVO EFFECTS OF c-MTIMP AND 6-MTI ON THE INCORPORATION OF GLYCINE-2-^{14}C INTO ACID-INSOLUBLE NUCLEIC ACIDS IN L1210/S

Drug	gly-2-^{14}C Incorporated (nmoles/ml cells)	Inhibition %
Control	42.7	0.0
MTI (82 mg/kg)	27.5	36.0
c-MTIMP (100 mg/kg)	28.7	33.0

Other details were described in Materials and Methods.

TABLE 9

IN VIVO EFFECTS OF c-MTIMP AND 6-MTI ON THE INCORPORATION OF GLYCINE-2-^{14}C INTO ACID-INSOLUBLE NUCLEIC ACIDS IN L1210/MTI

Drug	gly-2-^{14}C Incorporated (nmoles/ml cells)	Inhibition %
Control	55.3	0.0
MTI (82 mg/kg)	37.9	31.0
c-MTIMP (100 mg/kg)	36.3	34.0

Other details were described in Materials and Methods.

pathway into acid-insoluble nucleic acids was then measured.

The data in Table 9 show c-MTIMP and MTI do significantly inhibit glycine incorporation into acid-insoluble nucleic acids in L1210/MTI cells to almost the same degree as in L1210/S cells. This suggests that MTI itself may inhibit the PRPP amidotransferase in L1210 cells.

In this regard, there are possibilities that not only both MTI and MTIMP can inhibit PRPP amidotransferase; but also that MTIMP may cause another lethal toxicity to L1210 cells in some way while MTI does not.

Furthermore, the in vivo inhibitory effects of c-MTIMP also seem to be consistent with those associated with the in vitro feedback effects in L1210 cells.

INHIBITION OF DNA AND RNA SYNTHESIS IN L1210/MTI CELLS IN VITRO:

It has been believed that MTIMP primarily inhibits the PRPP amidotransferase in de novo purine biosynthesis. This incidence must, of necessity, affect the synthesis of DNA and RNA.

L1210 cells were suspended in Fischer medium with 1 mM MTI and c-MTIMP and were incubated at 37° C and the amount of incorporation of ^{3}H-TdR into DNA or ^{3}H-UR into RNA was measured. The data, shown in Figure 3, indicates that c-MTIMP does not significantly inhibit total DNA synthesis as well as does MTI. This results may imply that DNA synthesis was not influenced

FIGURE 3

INHIBITION OF DNA SYNTHESIS IN L1210/MTI CELLS IN VITRO

The reaction mixture consisted of 1 mM MTI, or c-MTIMP, 0.2 mM ^{3}H-TdR (5 µCi/umole) and Fischer medium in a total volume of 15 ml. The reaction flasks were incubated at 37° C in air; and at specified times 2 ml aliquots were delivered to chilled tubes containing 2 ml 10 % TCA. The precipitates were washed with 5 % TCA and solubilized in Protosol and counted as described in materials and Methods.

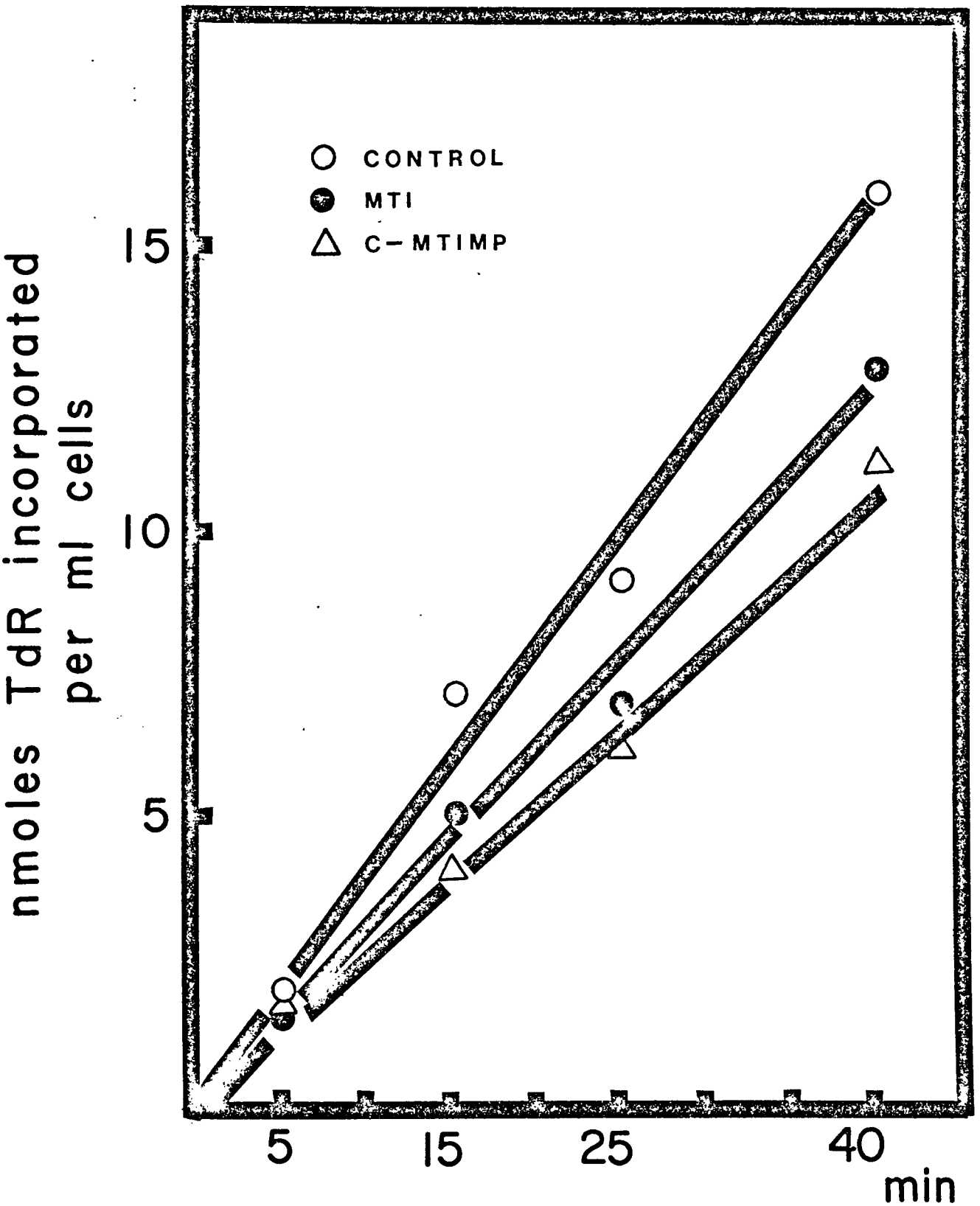

CONTROL
MTI
C-MTIMP
nmoles TdR incorporated per ml cells
15
10
5
5
15
25
40
min

FIGURE 4

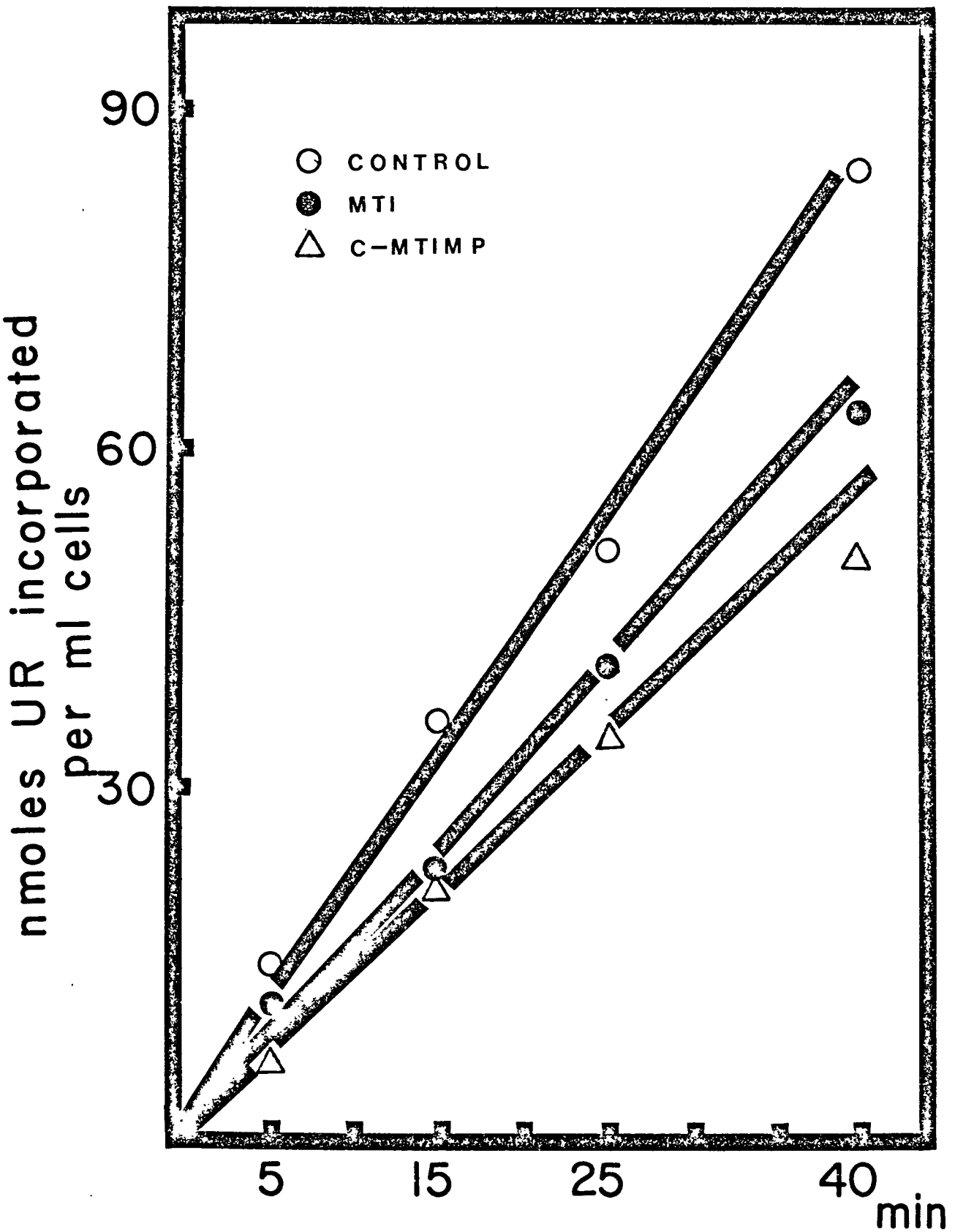
INHIBITION OF RNA SYNTHESIS

IN L1210/MTI CELLS IN VITRO

The reaction flask contained:

1 ml of L1210/MTI cells (2.8×10^8 cells)
1 mM MTI or c-MTIMP
0.2 mM ^{3}H-UR
and Fischer medium to give a final volume of 10 ml.

Other details are described in Materials and Methods.


90
60
30
CONTROL
MTI
C-MTIMP
nmoles UR incorporated per ml cells
5
15
25
40
min

by inhibition of the de novo purine biosynthesis during this relatively short period of time. But, RNA synthesis was considerably inhibited by c-MTIMP and MTI. It should be considered that the pool size of substrates for RNA synthesis may be directly influenced by the de novo purine biosynthetic pathwasy. Also, significant inhibition of RNA synthesis does not exclude the possibility that MTIMP may have other inhibitory effects (e.g., inhibition of polymerase system other than inhibition of PRPP amidotransferase).

ADENOSINE KINASE ACTIVITY IN L1210 CELLS:

In order to ensure that a deficiency of adenosine kinase activity obtained L1210/MTI cells, the enzyme activity was measured in whole cell preparations and cell-free extracts, with MTI-^{35}S as substrate. The data, shown in Table 10, indicates that L1210/S cells were able to convert MTI to MTIMP; whereas no measurable amount of MTIMP was detected in L1210/MTI in whole cell preparations. This result is consistent with cell-free extract experimentation. Therefore, it is reasonable to assume that the feedback inhibition by MTI in L1210/MTI cells was not due to the formation of MTIMP. In this regard, Henderson, et al. (46) found that 6-benzylthioguanine inhibited PRPP amidotransferase without the phosphorylation of its intact form.

TABLE 10

ADENOSINE KINASE ACTIVITY IN WHOLE CELL AND CELL-FREE EXTRACTS OF L1210/S AND L1210/MTI CELLS

Strain	Whole Cell Preparation MTIMP formed, umoles/ml	Cell-Free Extract
L1210/S	1.79	- -
L1210/MTI	NDA	NDA

NDA: No Detectable Amount.

For whole cell experiments, reaction tubes contained:

0.1 ml of 1 mM MTI-^{35}S (14,000 cpm/ml)
0.2 ml of L1210 cells (1:8 dilution, about 3.6 x 10^7 cells)
and 0.7 ml of calcium free Krebs-Ringer phosphate buffer with glucose to give a final volume of 1 ml.

Tubes were incubated at 37° C; a specified times, reaction was quenched with 0.2 ml of 2.6 N $HClO_4$ in cold and neutralized with KOH. After $KClO_4$ was discarded, 0.1 ml aliquots were spotted with carrier on Whatman # 3, developed with 5 % Na_2HPO_4 and UV spots were cut out and counted in 10 ml toluene.

For cell-free experiments:

0.15 ml of cell-free extract (1:4 dilution, 7.5 mg/ml protein),
0.10 ml of 10 mM MTI-^{35}S,
0.10 ml of ATP regenerating system
and calcium-free Krebs-Ringer phosphate buffer in glucose (5.5mM) were added to give a final volume of 0.5 ml. Tubes were incubated at 37° C; at specified time, reaction was quenched by immersion in boiling water for 3 minutes and chromatographed and counted as previously described.

SURVIVAL STUDY OF L1210/MTI STRAIN:

Female BDF_1 mice each received intraperitoneal implants of L1210/MTI cells, and 24 hours later 10 mg/kg MTI was intraperitoneally administered once daily for six consecutive days. Then, the average survival life span was compared with a control group which had been administered saline. As the data show in Table 11, the average life span of L1210/MTI strain was not increased by the administration of MTI, even though MTI may inhibit the PRPP amidotransferase.

Henderson, et al. (39) reported that primary growth inhibitory effects in Erhlich ascites cells by MTI results from the inhibition of PRPP amidotransferase. If this is so, it is reasonable to assume that L1210 cells may have some capacity for overcoming such feedback inhibition by MTI itself or the MTIMP form may inhibit some other lethal enzyme or enzymes.

INHIBITION OF DNA AND RNA POLYMERASES OF L1210/S CELLS:

According to unpublished personal observations (27), MTIMP is a potent inhibitor of purified DNA-directed RNA polymerase of E. coli. Therefore it is of interest to determine whether c-MTIMP and MTI inhibits the RNA and DNA polymerases of L1210 cells. As the data shows in Tables 10 and 11, neither c-MTIMP nor MTI significantly inhibited either of these enzyme systems.

TABLE 11

SURVIVAL STUDY OF L1210/MTI STRAIN

	No. of mice	Avg. weight	Average Survival (days)	Increase %
Saline	10	20 g	11 ± 0.1	0.0
MTI (10 mg/kg/day x6	10	20 g	12.2 ± 1	10.0

Female BDF_1 mice each received i.p. implants of 10^6 L1210/MTI cells. Treatment, started after 24 hours, was once daily for six consecutive days by i.p. injection.

TABLE 12

INHIBITION OF L1210/S RNA POLYMERASE

Drug	UTP Incorporated nmole/ml cells	Inhibition %
Control	2.43	0.0
MTI (2.5×10^{-4} M)	2.60	+ 7.0
c-MTIMP (2.5×10^{-4} M)	2.44	0.0

Broken cell preparations were used for the enzyme assays. The reaction tubes, in duplicates and complete except for substrates, were pre-incubated for 30 minutes at 37° C. Incubation was continued for an additional minutes after the addition of substrates. Other details are described in Materials and Methods.

TABLE 13

INHIBITION OF L1210/S DNA POLYMERASE

Drug	TTP Incorporated	Inhibition %
Control	4.51	0.0
MTI (10^{-4} M)	3.67	12.0
c-MTIMP (10^{-4}M)	3.59	20.0

Broken cell preparations were used for the enzyme assays. Reaction tubes, in duplicates and complete except for the substrates, were pre-incubated for 30 minutes and substrates were added and incubation was continued for an additional 20 minutes. Other details are described in Materials and Methods.

TABLE 14

INHIBITION OF

AMP PYROPHOSPHORYLASE ACTIVITY IN L1210/S AND L1210/MTI

		AMP Formed (cpm)	%
L1210/S	Control	2970	0.0
	MTI (10^{-3} M)	3630	+ 22.0
	MTIMP (10^{-3} M)	3660	+ 28.0
L1210/MTI	Control	1700	0.0
	MTI (10^{-4} M)	1900	+ 15.0
	MTIMP (10^{-4} M)	2070	+ 22.0

One ml of reaction mixture consisted of:

0.2 ml cell-free extract (400 ug protein),
Tris buffer, pH 7.6, 0.1 M,
Adenine-8-^{14}C, 0.5 mM,
PRPP, 2 mM
and drugs.

Incubation time was 20 minutes

FIGURE 5

HYDROLYSIS OF c-MTIMP AND c-AMP BY

CELL-FREE EXTRACTS OF L1210/MTI CELLS

Reaction tubes contained:

0.2 ml of cell-free extract of L1210/MTI cells (1:4 dilution - approximately 7.5 mg/ml protein)
and 0.2 ml of 10 mM cAMP or c-MTIMP in calcium-free Krebs-Ringer phosphate buffer
to a final volume of 1.4 ml.

The tubes were incubated at 37° C for a specified time; the reaction was stopped by immersion in boiling water for 3 minutes. After denatured protein was removed by centrifugation 100 ul of aliquots were spotted on Whatman # 3 paper and developed with isopropyl alcohol: 17 N ammonium hydroxide: 0.1 M boric acid (60:30:10); UV absorption spots were cut out and eluted with water and the O. D. of each was determined spectrophotometrically at 265 mu and 292 mu.

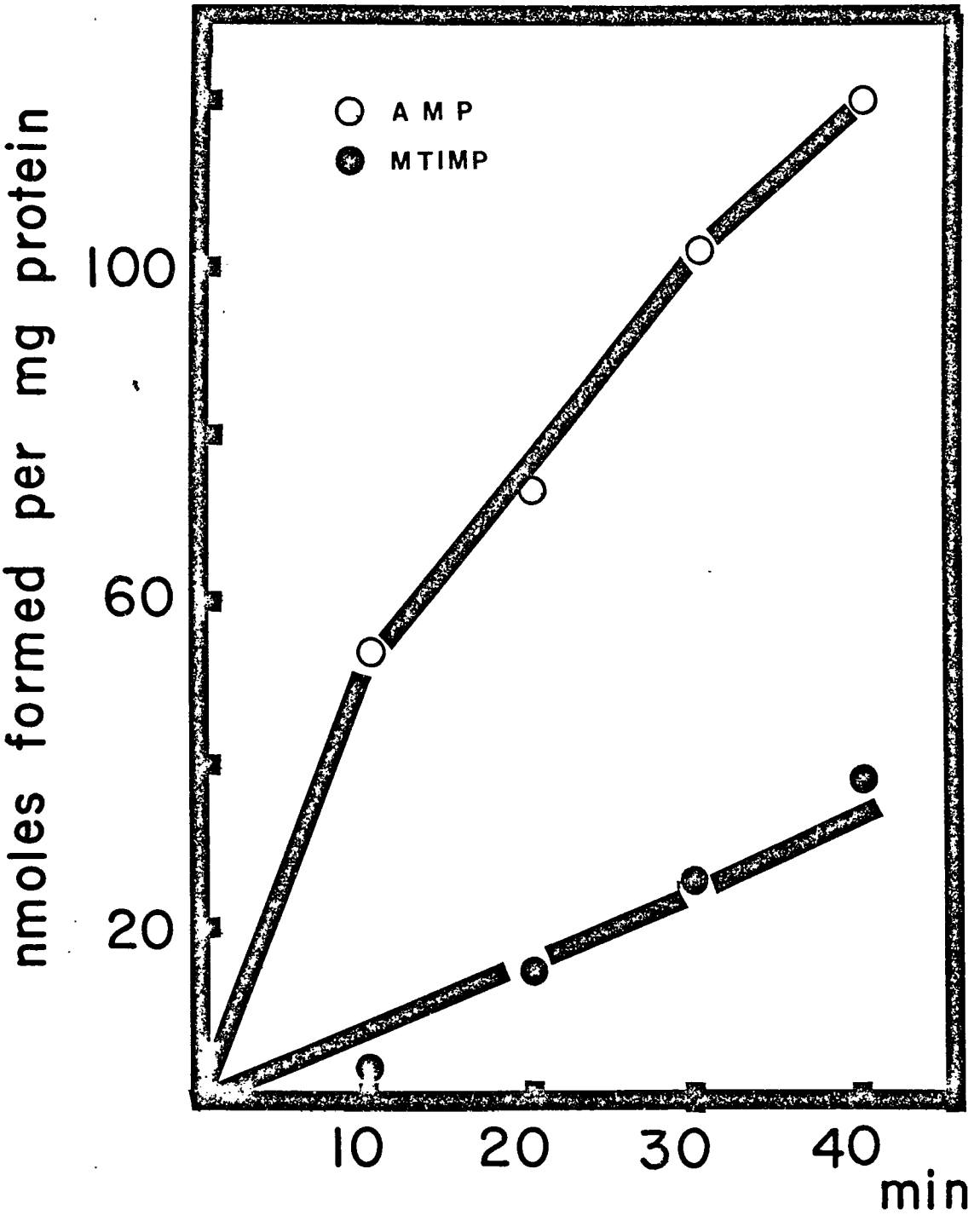
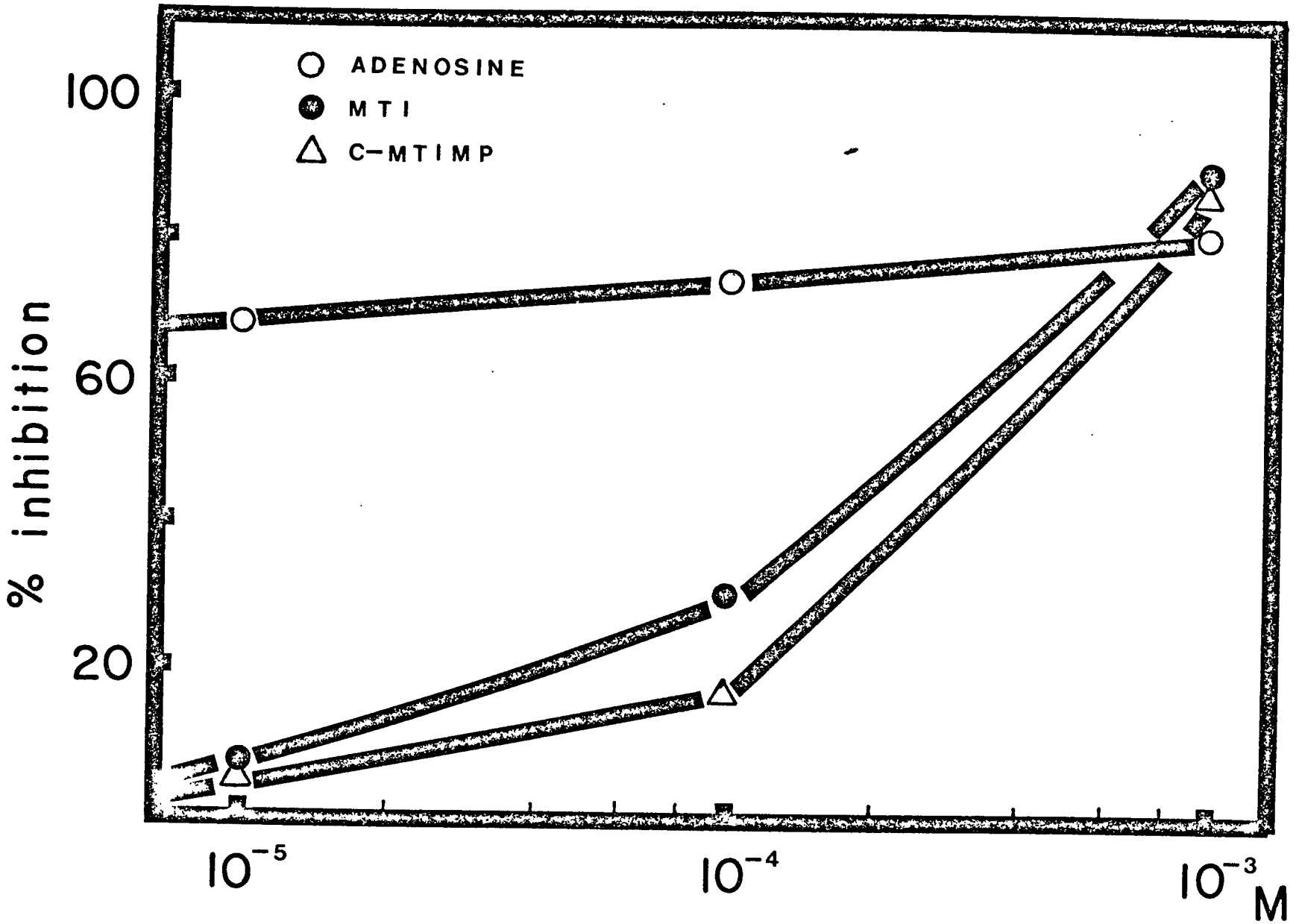

AMP
MTIMP
nmoles formed per mg protein
100
60
20
10
20
30
40
min

FIGURE 6

INHIBITION OF FGAR FORMATION BY VARIOUS CONCENTRATIONS OF INHIBITOR

L1210/S cells were incubated at 37° C with 10^{-5} M. 10^{-4} M and 10^{-3} M adenosine, MTI, and c-MTIMP. Other details were given in Materials and Methods.


ADENOSINE
MTI
C-MTIMP
100
60
20
% inhibition
10^{-5}
10^{-4}
10^{-3} M

INHIBITION OF AMP PHYROPHOSPHORYLASE IN L1210 CELLS:

It has been suggested that AMP pyrophosphorylase and GMP-IMP pyrophosphorylase are essential enzymes in the purine nucleotide metabolism of rapidly growing tissue (41). Bennett, et al. (40) found that 6-MP inhibits the interconversion of preformed purine nucleotides in L1210, Erhlich ascites cells, and Sarcoma 180 cells. Furthermore, Murray (42, 43) observed that AMP pyrophosphorylase was inhibited by AMP; If MTIMP functions as an analog of AMP it may inhibit this enzyme. It was, therefore, decided to study the effects of c-MTIMP and MTI on this enzyme system. The data in Table 12 shows no significant inhibitory effects were exhibited.

HYDROLYSIS OF c-MTIMP AND c-AMP BY CELL-FREE EXTRACTS OF L1210/MTI CELLS:

In order to ascertain whether c-MTIMP was a substrate of cyclic phosphodiesterase in L1210 cells, c-MTIMP and c-AMP were incubated with cell-free extracts of L1210/MTI cells in calcium-free Krebs-Ringer phosphate buffer. As the data shown in Figure 5 indicates, the rate of hydrolysis of c-MTIMP was much lower than that of c-AMP. This results implies that c-MTIMP may give prolonged drug effects in vivo.

INHIBITION OF FGAR FORMATION BY VARIOUS CONCENTRATIONS OF INHIBITORS:

In order to obtain the relative potencies of adenosine, MTI, and c-MTIMP, concentration curves were made. As is shown in Figure 6, the concentration ranged from 10^{-3} M to 10^{-5} M. The potency of feedback inhibition by adenosine only decreased by about 10 %; whereas, inhibition by MTI or c-MTIMP was markedly decreased (lower than 10^{-3} M). These results suggest that if the concentration of MTI decreased to a certain level, the de novo purine biosynthetic pathway would be operative in a manner that can be equated almost with normalcy. It is well known that nucleotides do not readily penetrate the cell membrane; whereas nucleosides are readily transported across the cell membrane. Therefore, in light of the mechanism of resistance to MTI in L1210 cells, it is possible that the concentration of MTIMP formed by adenosine kinase would be retained for prolonged periods of time within the cell. However, MTI would be excreted so rapidly, its effects of an inhibitory nature are diminished rapidly. Therefore, L1210 cells resistant to MTI due to the loss of adenosine kinase are able to survive eventhough MTI itself inhibits the PRPP amidotranferase in the de novo purine biosynthetic pathway.

DISCUSSION

It has been generally agreed that MTI inhibits tumor cell growth by being phosphorylated to MTIMP, which inhibits PRPP amidotransferase. It is also generally agreed that the conversion of MTI to MTIMP by adenosine kinase is a prerequisite process in order that feedback inhibition is demonstrated. Tumor cell lines that are resistant to MTI show marked deficiencies of adenosine kinase activity.

In order to circumvent the resistance due to the loss of adenosine kinase, the present studies were conducted: to synthesize c-MTIMP and to evaluate its biochemical effect on Erhlich and L1210 cells resistant to MTI.

Feedback inhibition studies suggested that c-MTIMP entered the cell and was hydrolyzed to MTIMP by cyclic phosphodiesterase. This observation is based upon the formation of MTIMP from c-MTIMP by bovine cyclic phosphodiesterase and extracts of L1210 cells. Furthermore, c-MTIMP does significantly inhibit the PRPP amidotransferase in EAC/S, EAC/MTI, L1210/S and L1210/MTI cell lines.

Theophylline which is an inhibitor of cyclic phosphodiesterase relieved the feedback inhibition of c-MTIMP. This result

substantiates the reasoning that the conversion of c-MTIMP to MTIMP is mediated by some cyclic phosphodiesterase.

In addition, according to survival studies, L1210/MTI strains do not show increased survival time with the treatment of MTI. If MTI itself is an inhibitor of PRPP amidotransferase, as was strongly suggested in L1210/MTI cells, some unknown mechanism of resistance apparently exixts in L1210 cells.

Probable enzymes which might be inhibited by MTIMP were studied. DNA and RNA polymerases and AMP pyrophosphorylase were not significantly inhibited by c-MTIMP or MTI.

The feedback inhibitory effect of c-MTIMP was compared with that of adenosine and MTI. However, the comparison could only be qualitative. For the quantitative comparison, the rate of entry of c-MTIMP must be measured. Adenosine shows potent feedback inhibition in both EAC/MTI and L1210/MTI, although no measurable amount of MTIMP is formed in the whole cell preparations or cell-free extracts for the adenosine kinase assay.

These results suggest that adenosine kinase has apparently mutated and is still sensitive to adenosine, but not to MTI due to continuous suppression with MTI. This observation is supported by the similar findings of Henderson, et al. (44). However, PRPP amidotransferase in L1210/MTI cells still exhibits sensitivity to MTI itself.

In order to determine the relative potencies of adenosine, MTI and c-MTIMP with given assay conditions, concentration curves were made, which showed that the feedback inhibitory effects of MTI and c-MTIMP below 10^{-3} M decreased rapidly; whereas, the inhibitory effect of adenosine was almost the same throughout the concentration range of 10^{-3} M to 10^{-5} M.

This may reflect that L1210/MTI cells may more rapidly excrete MTI than MTIMP. This possibility may be supported by the fact that the nucleotides do not readily penetrate the cell membrane, whereas, nucleosides freely penetrate the cell membrane. Therefore, L1210/MTI can be resistant to MTI.

Finally, several other interesting areas remain to be investigated:

1. Inhibition study of MTI and purified PRPP amidotransferase of L1210 cells.
2. Measurement of rate of entry of c-MTIMP.
3. Long-term in vivo effects of c-MTIMP.
4. Binding studies of MTI, MTIMP and TIMP (Do these inhibitors bind to the same inhibitor site on PRPP amidotransferase, such as on GMP site or on AMP site?).

SUMMARY

In order to circumvent resistance to MTI, c-MTIMP was synthesized and biochemical studies with c-MTIMP were made with L1210 cells and Erhlich ascites cells. The feedback inhibition studies showed c-MTIMP to be a potent inhibitor of PRPP amidotransferase in both the sensitive and resistant cell lines. Its feedback inhibition may be due to the conversion of c-MTIMP to MTIMP by cyclic phosphodiesterase.

L1210/MTI cells were sensitive to MTI although no adenosine kinase activity was measurable. Both cell lines were sensitive to adenosine.

MTI and c-MTIMP did not inhibit DNA or RNA polymerases or AMP pyrophosphorylase of L1210 cells. MTI and c-MTIMP did not give rise to significant feedback inhibition at concentrations less than 10^{-3}M; whereas, the inhibition by adenosine did not decrease over a concentration range from 10^{-3}M to 10^{-5}M.

The possible mechanism of resistance in L1210/MTI could be due, at least in part, to the rapid excretion of MTI by L1210/MTI cells.

REFERENCES

1. Kidder, G. W., V. C. Dewy, R. E. Parks, Jr., and G. L. Woodside, Science, 109, 511 (1949).
2. Kidder, G. W. and V. C. Dewy, J. Biol. Chem., 179, 181 (1949).
3. Jacques, J. A. and D. J. Hutchison, Cancer Res., 19. 397. (1957).
4. Wolpert, M. K., S. P. Damile, J. E. Brown, E. Szny, K. C. Agrawal and A. C. Sartorelli, Cancer Res., 31, 1620. (1971).
5. Henderson, J. F., I. C. Caldwell and A. R. P. Paterson, Cancer Res., 27, 1773 (1967).
6. Sartorelli, A. C. and G. A. LePage, Cancer Res., 18, 457 (1957)
7. Fox, J. J., I. Wempen, A. Hampton and J. L. Doerr, J. Amer. Chem. Soc., 80, 1669 (1958).
8. Reist, E. J., H. B. Z. Goodman, G. R. Baker and W. W. Lee, J. Org. Chem., 27, 3274 (1962)
9. Don, K. and K. Gerzon, J. Med. Chem., 8, 275 (1965).
10. Claton, S. J. and W. E. Fitzgibbon, Jr., Synthetic Procedures in Nucleic Acid Chemistry, p. 279 (1968).

11. Tener, G. M., J. Amer. Chem. Soc., 83, 159 (1961).

12. Montgomery, J. A. and H. J. Thomas, J. Org. Chem., 26, 1926 (1961).

13. Smith, M., G. L. Drummond and H. G. Hkorana, J. Amer. Chem. Soc., 83, 698 (1961).

14. Thomas, H. J., and J. A. Montgomery, J. Med. Chem., 11, 44 (1968).

15. Gulyassy, P. F. and R. L. Oken, Proc. Soc. Exptl. Biol. Med., 137, 261 (1971).

16. Randerath, K. and E. Randerath, J. Chromatog., 16, 111 (1964).

17. Moffat, J. D. and H. G. Khorana, J. Amer. Chem. Soc., 83, 649 (1961).

18. Spoor, T. C., J. E. Evans and A. P. Kimball, Proc. Soc. Exp. Biol. Med., 136, 605 (1971).

19. Brockman, R. W., Mechanisms of Resistance to Anticancer Agents, Advances in Cancer Research, Academic Press, Inc., N. Y., p. 129 (1963).

20. Wyngaarden, J. B. and D. M. Ashton, J. Biol. Chem., 234, 1492 (1959).

21. McCollister, R. J., W. R. Gillbert, Jr., D. M. Ashton and J. B. Wyngaarden, J. Biol. Chem., 239, 1560 (1964).

22. Brockman, R. W., Exploitable Molecular Mechanisms and Neoplasia, Williams and Wilkins Co., Baltimore, p. 435 (1968).

23. Dawson, R. M. C., D. C. Elliott, W. H. Elliott and K. M. Jones, Data for Biochemical Research, Oxford University Press, London, p. 208 (1959).

24. Haley, E. E., G. A. Fischer and A. D. Welch, Cancer Res., 21, 532 (1961).

25. LePage, G. A. and M. Jones, Cancer Res., 21, 642 (1961).

26. Henderson, J. F., J. Biol. Chem., 237, 2631 (1962).

27. Nixon, J., PhD Dissertation, University of Houston, Houston (1972).

28. Tomisek, A. T., H. J. Kelley and H. E. Skipper, Arch. Biochem. Biophys., 64, 437 (1956).

29. Lowry, O. H., J. J. Rosenbrough, A. L. Farr and R. J. Randall, J. Biol. Chem., 193, 265 (1951).

30. Kimball, A. P. and M. W. Wilson, Proc. Soc. Exp. Biol. Med., 127, 429 (1968).

31. Vander Velde, G., P. R. Loy and A. P. Kimball, Proc. Soc. Exp. Biol. Med., 132, 831 (1970).

32. Pierre, K. J. and G. A. LePage, Proc. Soc. Exp. Biol. Med., 127, 432 (1968)

33. Moravek, J. and Z. Nejedly, Chem. and Indust., p. 530 (1960).

34. Caldwell, I. C., J. F. Henderson and H. R. P. Paterson, Can. J. Biochem., 44. 229 (1966).

35. Brockman, R. W., G. G. Kelley, D. Stutts and V. Copeland, Nature, 191, 469 (1961)

36. Bennett, Jr., L. L., H. P. Schneibli, M. H. Vail, P. W. Allan and J. A. Montgomery, Molecular Pharmacology, 2, rew (1966).

37. Robinson, G. A., R. W. Butcher and G. W. Sutherland, Cyclic AMP, Ann. Rev. Biochem., 31, 149 (1968).

38. Schroder, J. and P. G. W. Plagemann, Cancer Res., 32, 1082 (1972).

39. Shantz, G. D., L. J. Fontenelle and J. F. Henderson, Biochem. Pharmaco., 21, 1203 (1972).

40. Bennett, Jr., L. L., L. Simpson, J. Golden and T. L. Barker, Cancer Res., 23, 1574 (1963).

41. Murray, A. W., Biochem. J., 100, 671 (1966).

42. Murray, A. W., Biochem. J., 100, 275 (1966).

43. Murray, A. W., Biochem. J., 100, 664 (1966).

44. Paterson, A. R. P. and M. C. Wang, Cancer Res., 30, 2379 (1970).

45. Bennett, Jr., L. L., R. W. Brockman, H. P. Schnebli, S. Chumley, G. J. Dixon, F. M. Schabel, Jr., E. A. Dulmadge, H. E. Skipper, J. A. Montgomery and H. J. Thomas, Nature, 205, 1276 (1965).

46. Henderson, J. F. and M. K. Y. Khoo, J. Biol. Chem., 240, 3104 (1965).

47. Hill, D. L. and L. L. Bennett, Jr., Biochem., 8, 122 (1969).

48. LePage, G. A. and E. M. Hersh, Biochem. Biophy. Res. Comm., 40, 1918 (1972).

49. Meyer, R. B., S. R. K. Robins, R. J. Bauer, M. K. Dimmitt and L. N. Simon, Biochem., 2, 2704 (1972).

50. Lomax, C. A. and J. F. Henderson, Can. J. Biochem., 50, 423 (1972).

51. Brockman, R. W. and C. Chumley, Biochim. Biophy. Acta, 95, 365 (1965).

52. Caldwell, I. C., J. F. Henderson and A. K. P. Paterson, Can. J. Biochem., 45, 735 (1967).

53. Hampton, A., J. J. Biesel, A. E. Moor and G. B. Brown, J. Amer. Chem. Soc., 78, 5695 (1956).