MOLECULAR PHARMACOLOGY, 24:109–114

MOL PHARM

Incorporation of 5-Aza-2′-Deoxycytidine-5′-Triphosphate into DNA

Interactions with Mammalian DNA Polymerase α and DNA Methylase

JACQUES BOUCHARD[1] AND RICHARD L. MOMPARLER

Département de Pharmacologie, Université de Montréal, and Centre de Recherche Pédiatrique, Hôpital Ste-Justine, Montréal, Québec, Canada H3T 1C5

Received December 21, 1982; Accepted March 28, 1983

SUMMARY

In order to understand further the molecular mode of action of 5-Aza-2′-deoxycytidine (5-AZA-dCyd), a potent antileukemic agent, we prepared enzymatically 5-Aza-2′-deoxycytidine 5′-triphosphate (5-AZA-dCTP) and performed studies with purified DNA polymerase α and DNA methylase from mammalian cells. DNA polymerase α catalyzed the incorporation of 5-AZA-dCTP into DNA. The apparent K_m value for 5-AZA-dCTP was estimated to be 3.0 μM; the K_m of dCTP was 2.0 μM. The apparent V_{max} of 5-AZA-dCTP was slightly lower than that for dCTP. 5-AZA-dCTP was a weak competitive inhibitor (K_i 4.3 μM) with respect to dCTP. Template studies with 5-AZA-dCTP showed that this nucleotide analogue was incorporated into poly(dIC), but not into poly(dAT), suggesting that the incorporation follows the rules of Watson-Crick base pairing. Incorporation of 5-AZA-dCTP into hemimethylated DNA produced a significant inhibition of DNA methylase. These results show that 5-AZA-dCTP is a very good substrate for DNA polymerase α and that its incorporation into DNA inhibits DNA methylation.

INTRODUCTION

5-AZA-dCyd[2] is a nucleoside analogue of deoxycytidine differing only by the replacement of carbon atom 5 by a nitrogen atom. It is an effective antileukemic agent in animal models (1–3) and in humans (4). The antineoplastic action of 5-AZA-dCyd can be reversed by deoxycytidine (1, 5). The active metabolite is the nucleotide derivative, since cells deficient in deoxycytidine kinase are resistant to 5-AZA-dCyd (6). It has been proposed that the lethal action of 5-AZA-dCyd is due to its incorporation into DNA (2, 7), possibly producing an interference in DNA function due to the chemical instability of the nucleoside analogue (8).

Recent reports indicate that 5-AZA-dCyd incorporation into DNA can induce cellular differentiation by producing some degree of hypomethylation of cytosine in DNA (9–11). In mammalian cells, about 3% of the cytosine moieties in DNA are present at 5-methylcytosine, with methylation taking place immediately after DNA replication (12). Currently, it is proposed that the degree of DNA methylation is related to gene expression (12). It has been suggested that 5-AZA-dCyd incorporation into DNA can inhibit DNA methylase (9, 10, 12, 13).

In this report, in order to understand the mechanism of action of 5-AZA-dCyd, we have investigated the interaction of its triphosphate derivative, 5-AZA-dCTP with a mammalian DNA polymerase. We have also investigated the incorporation of 5-AZA-dCTP into DNA and its relationship to template activity for DNA methylase, an enzyme that uses *S*-adenosylmethionine as a methyl donor.

Our results indicated that 5-AZA-dCTP is as good a substrate as dCTP for the DNA polymerase. The *in vitro* system used to study the methylation showed that a small number of 5-azacytosine residues present in DNA can significantly inhibit DNA methylase. The results presented here with purified enzymes are consistent with the reports on cellular studies that 5-AZA-dCyd is readily incorporated into DNA (2, 14) and produces hypomethylation (9, 10, 13).

MATERIALS AND METHODS

Nonradioactive nucleotides were obtained from P-L Biochemicals (Milwaukee, Wisc.) Radioactive nucleotides and *S*-adenosyl-L-[*methyl*-^{3}H]methionine were purchased from New England Nuclear of Canada (Montreal). 5-AZA-dCyd was synthesized by Dr. A. Pískala and obtained from Chemapol (Prague, Czechoslovakia). [^{3}H]5-AZA-dCyd (20

This investigation was supported by Grant MA-6356 from the Medical Research Council of Canada and by LEUCAN.

[1] Recipient of a Studentship from the Fonds de la Recherche en Santé du Québec.

[2] The abbreviations used are: 5-AZA-dCyd, 5-Aza-2′-deoxycytidine; 5-AZA-dCMP, 5-Aza-dCyd-5′-monophosphate; 5-AZA-dCTP, 5-Aza-dCyd-5′-triphosphate; DNAse, deoxyribonuclease I.

0026-895X/83/040109-06$02.00/0
Copyright © 1983 by The American Society for Pharmacology and Experimental Therapeutics.
All rights of reproduction in any form reserved.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

Ci/mmole) was a gift from Dr. J. Veselý. Poly(dAT), nucleosidemonophosphate kinase (ATP:nucleosidemonophosphate phosphotransferase, EC 2.7.4.4), nucleoside 5′-diphosphate kinase (ATP:nucleosidediphosphate phosphotransferase, EC 2.7.4.6), *Escherichia coli* DNA polymerase I (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7), and *S*-adenosylmethionine were obtained from Boehringer Mannheim Canada (Montreal). Poly(dIC), DNA from calf thymus, and *Micrococcus luteus* were purchased from Miles Biochemicals (Rexdale, Ont.). The cation-exchange resin AG-1X8 and Bio-Gel P-2 were supplied by Bio-Rad Laboratories (Mississauga, Ont.). DEAE-cellulose Whatman DE53 was obtained from Mandel Scientific (Rockwood, Ont.). DEAE-Sephadex A-25 and Sephadex G-25 were supplied by Pharmacia Canada Ltd. (Montreal). Deoxyribonuclease I (EC 3.1.4.5) was purchased from Worthington Biochemicals (Freehold, N.J.).

DNA polymerase α from calf thymus was partially purified by a modification of the method of Momparler *et al.* (15). The DEAE-cellulose step was omitted. The specific activity was about 2.2 units/mg. One unit of enzyme activity was defined as the amount of enzyme catalyzing the incorporation of 1.0 nmole of radioactive nucleotide into an acid-insoluble product per 10 min at 37°. More than 99% of the enzyme activity was inhibited by *N*-ethylmaleimide (10 mM) or NaCl (250 mM), indicating nonsignificant contamination with DNA polymerases β and γ.

DNA methylase (*S*-adenosylmethionine:DNA-cytosine 5-methyltransferase, EC 2.1.1.37) was purified from L1210 leukemic cells by salt extraction of isolated nuclei and DEAE-cellulose chromatography (16).

DNA polymerase assay. DNA polymerase activity was assayed by the filter disc method (17), using Whatman GF/C glass-fiber discs (2.4-cm diameter). The composition of the reaction mixture is given in the legends to the tables and figures. The reaction mixture was diluted with 3.0 ml of cold 5% trichloroacetic acid, placed on the filters, and washed twice with cold 5% trichloroacetic acid and once with ice-cold ethanol. The disc was then dried at 50° and placed in a toluene-based scintillant for radioactivity analysis. Denatured DNA was obtained by heating a solution of native calf thymus DNA [1.0 mg/ml in 10 mM NaCl and 1.0 mM EDTA (pH 8.0)] at 100° for 15 min and placing it immediately on ice. Under these conditions a hyperchromic shift of 35% at 260 nm was attained.

DNA methylase assay. DNA methylase activity was assayed by using specific DNA templates. In a reaction mixture of 50 μl, 11.5 μg of DNA methylase were incubated with the DNA template, 0.45 nmole of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1 μCi), 2.5 μmoles of Tris-HCl (pH 7.5), 2.5 μmoles of EDTA, 16 μg of bovine serum albumin, and 0.5 μmole of β-mercaptoethanol at 37° for the indicated time and assayed using the GF/C filter method described above. Treatment of the radioactive product of the reaction with deoxyribonuclease I, RNAse, or proteinase K (Beckman Instruments, Palo Alto, Calif.) showed that >95% of radioactivity incorporated was present in DNA.

Preparation of DNA. M. luteus DNA used in methylation experiments was treated with proteinase K and extracted with phenol-chloroform. This purified DNA was used as a template for DNA methylase and in the nick-translation (18) experiments.

Synthesis of 5-AZA-dCTP. 5-AZA-dCTP was synthesized enzymatically from 5-AZA-dCyd. 5-AZA-dCMP was synthesized first using deoxycytidine kinase (NTP:deoxycytidine 5′-phosphotransferase, EC 2.7.1.12) partially purified from calf thymus (19). The reaction mixture (5 ml) contained the following: Tris-HCl buffer, 50 mM (pH 7.4); ATP, 5 mM; $MgCl_2$, 5 mM; 5-AZA-dCyd, 10 μmoles; the enzyme, 6.0 units; and 10% (v/v) glycerol. The reaction mixture was incubated for 2 hr at 37° and deproteinated by ice-cold ethanol (10 ml). 5-AZA-dCMP was purified by column chromatography on AG-1X8 resin (0.8 cm^2 × 35 cm) using a linear gradient of NaCl (0–800 mM) containing 5 mM Tris-HCl (pH 7.4). 5-AZA-dCMP was concentrated on a small column of DEAE-Sephadex A-25 (0.4 cm^2 × 4 cm) and desalted by column chromatography on Bio-Gel P-2 (0.6 cm^2 × 54 cm). The final yield of this procedure was about 20%. Purified 5-AZA-dCMP was then incubated with nucleosidemonophosphate kinase and nucleosidediphosphate kinase for 3 hr at 25°. The reaction mixture (2 ml) contained ATP, 5 mM; $MgCl_2$, 5 mM; Tris-HCl, 50 mM (pH 7.4); nucleosidemonophosphate kinase, 2.5 units; and nucleosidediphosphokinase, 4.0 units. The reaction was terminated by the addition of ice-cold ethanol (2 ml). The final product, 5-AZA-dCTP, was purified by column chromatography as described above for 5-AZA-dCMP. The yield of the synthesis from 5-AZA-dCMP was estimated to be about 45%. [^{3}H]5-AZA-dCTP was also synthesized enzymatically using radiolabeled [^{3}H]5-AZA-dCyd. The total synthesis was made in one step with the three kinases in the same reaction mixture described above, and the [^{3}H]5-AZA-dCTP was purified on a small column of AG-1X8 resin. The final yield of this synthesis was about 50%.

Synthesis of hemimethylated DNA. The nick-translation reaction (18) was used to incorporate 5-methyl-dCTP or 5-AZA-dCTP into the DNA of *M. luteus.* In this reaction, traces of DNAse I nicks the DNA, and *E. coli* DNA polymerase I incorporates the deoxynucleotides into DNA in a repair-type reaction. In typical experiment, 7 μg of purified *M. luteus* DNA; 10 mM Tris-HCl (pH 7.8); 5 mM MgCl; 20 μM each of dATP, dGTP, and dTTP; 2 mM β-mercaptoethanol; 2.5 units of *E. coli* DNA polymerase I; 5 pg of DNAse I; and 100 μM 5-methyl-dCTP in 50 μl were incubated at 14.5° for 60 min. At the end of this period, template activity of this DNA was determined by adding 5.0 mM EDTA, 9 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1 μCi), and DNA methylase (11.5 μg) to the reaction mixture (final volume: 77.5 μl). The incubation was then continued for 90 min at 37°, and the acid-insoluble radioactivity was determined on GF/C filters.

Synthesis of hemimethylated DNA that contained 5-AZA-dCyd residues. Two successive nick-translation reactions were performed to produce a hemimethylated DNA that contained 5-azacytosine moieties (18). After the first nick-translation reaction as described above, the 5-methyl-dCTP was removed from the reaction mixture by spin dialysis (20) through a small column of Sephadex G-25 (0.5 ml); a second reaction of nick-translation was performed on this DNA. The reaction mixture (50 μl) contained 3.5 μg of hemimethylated DNA; 10 mM Tris-HCl (pH 7.8); 5 mM MgCl; 20 μM each of dATP, dGTP, and dTTP; various concentrations of 5-AZA-dCTP or dCTP (control); 2.5 units of *E. coli* DNA polymerase I; and 5 pg of DNAse I. After incubation at 14.5° for 30 min, 20 μl EDTA (200 mM), DNA methylase (11.5 μg of protein), and 0.45 μmole of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1 μCi) were added to determine the methylase template activity of the DNA. The reaction mixture was incubated for 90 min at 37°, and the acid-insoluble radioactivity was determined on GF/C filters.

RESULTS

Kinetic studies on 5-AZA-dCTP and DNA polymerase α. The effect of 5-AZA-dCTP on the reaction rate of DNA polymerase α in the presence of different concentrations of [^{3}H]dCTP is shown in Fig. 1. The data have been plotted according to the method of Lineweaver and Burk (21). The inhibition produced by 5-AZA-dCTP appeared to be competitive with respect to [^{3}H]dCTP. The apparent K_m for [^{3}H]dCTP incorporation was estimated to be 2.0 μM. The apparent K_i for 5-AZA-dCTP was 3.4 μM. The apparent V_{max} for the reaction using [^{3}H]dCTP as the reactive substrate was about 5500 pmoles incorporated/20 min/mg of protein.

The effect of dCTP on the reaction rate in the presence of different concentrations of [^{3}H]5-AZA-dCTP is shown in Fig. 2. The inhibition produced by dCTP appeared to be competitive with respect to [^{3}H]5-AZA-dCTP. The apparent K_m for [^{3}H]5-AZA-dCTP and K_i for dCTP obtained from this plot were estimated to be 3.0 and 1.6 μM, respectively. The apparent V_{max} for the reaction using [^{3}H]5-AZA-dCTP as the radioactive substrate was 4630 pmoles incorporated/20 min/mg of protein. A summary of the kinetic data obtained from three or more determinations is presented in Table 1.

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

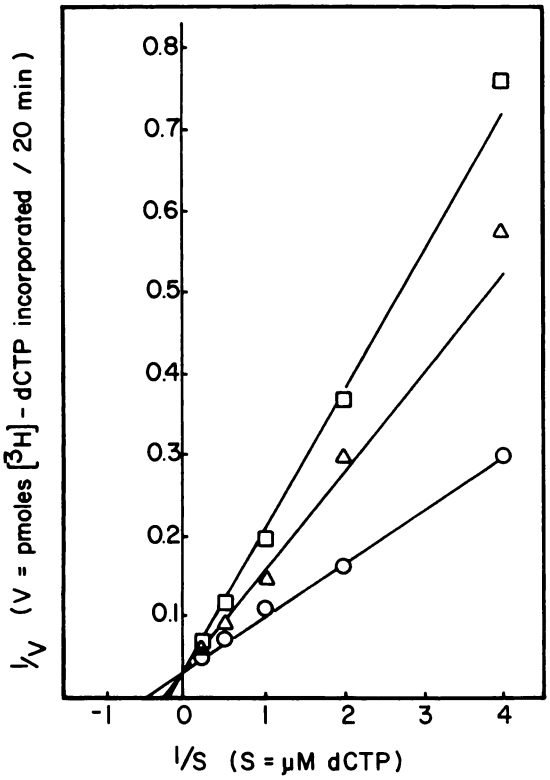

FIG. 1. *Effect of 5-AZA-dCTP on reaction rate in the presence of different concentrations of [^{3}H]dCTP*

The reaction mixture (50 μl) contained 10 μmoles of potassium phosphate (pH 7.2); 0.3 μmole of $MgCl_2$; 10 μg of denatured DNA; 2.5 nmoles each of dATP, dGTP, and dTTP; 0.01 unit of DNA polymerase from calf thymus; and the indicated concentrations of [^{3}H]dCTP (8.5 × 10^4 cpm) and 5-AZA-dCTP. The mixture was incubated at 37° for 20 min and assayed as described under Materials and Methods. ○, No 5-AZA-dCTP; △, 5-AZA-dCTP, 5 μM; □, 5-AZA-dCTP, 10 μM.

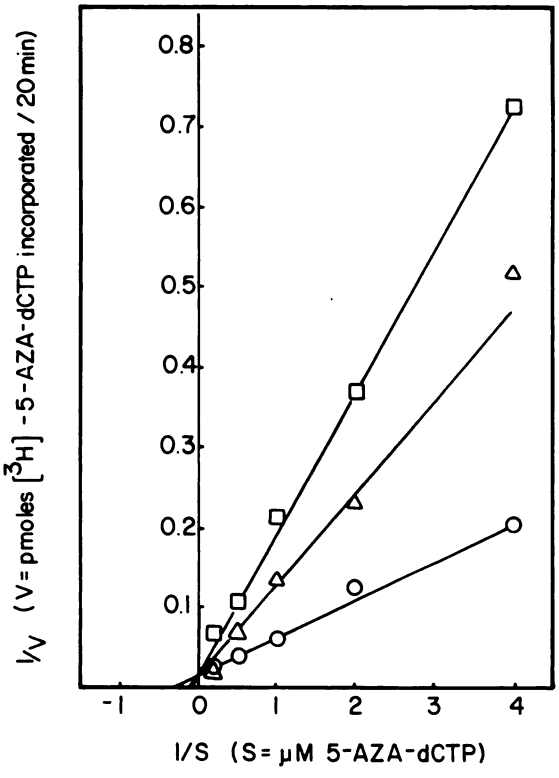

FIG. 2. *Effect of dCTP on reaction rate in the presence of different concentrations of [^{3}H]5-AZA-dCTP*

The reaction mixture (50 μl) contained 7.5 μmoles of potassium phosphate (pH 7.2); 0.3 μmole of $MgCl_2$; 10 μg of denatured DNA; 2.5 nmoles each of dATP, dGTP, and dTTP; 0.03 unit of DNA polymerase from calf thymus; and the indicated concentrations of [^{3}H]5-AZA-dCTP and dCTP. The mixture was incubated at 37° for 20 min and assayed as described under Materials and Methods. ○, No dCTP; △, dCTP, 5 μM; □, dCTP, 10 μM.

Template requirements for the incorporation of 5-AZA-dCTP into DNA. The template requirements for [^{3}H]5-AZA-dCTP, [^{3}H]dCTP, and [^{3}H]dTTP incorporation were investigated using the two synthetic polydeoxynucleotides, poly(dIC) and poly(dAT) (Table 2). These experiments showed that [^{3}H]5-AZA-dCTP incorporation into poly(dAT) was negligible as compared with its incorporation into poly(dIC). [^{3}H]dCTP incorporation was similar to the incorporation of [^{3}H]5-AZA-dCTP for these two synthetic polydeoxynucleotides. [^{3}H]dTTP was incorporated into poly(dAT), but not into poly(dIC).

Hemimethylated DNA is a better template for DNA methylase activity. Since hemimethylated DNA is the most active template for DNA methylase (22), we used the nick-translation reaction (18) to incorporate 5-methyl-dCTP into DNA. *Micrococcus luteus* DNA was used in this reaction because of its high GC content (70%). As shown in Fig. 3, after the incorporation of 5-methyl-dCTP into DNA, there was a significant increase in the methyl acceptability of the template. Under the experimental conditions used, the optimal amount of 5-methyl-dCTP incorporated into DNA that gave the maximal methyl acceptor activity occurred after 50 min of incubation with DNAse I and *Escherichia coli* DNA polymerase I. Increasing the incubation time beyond 60 min resulted in a loss of methyl acceptor activity of the DNA.

5-AZA-dCTP incorporation into hemimethylated DNA inhibited DNA methylase activity. The results obtained, when hemimethylated DNA that contained 5-azacytosine was used as a template for DNA methylase, are summarized in Table 3. A schema for the experimental plan is shown in Fig. 4. The first nick-translation reaction was used to incorporate 5-methyl-dCTP to synthesize hemimethylated DNA. This DNA was used as a template for a second nick-translation for 5-AZA-dCTP incorporation. This hemimethylated DNA that contained 5-azacytosine residues was then tested for its template activity for DNA methylase. The incorporation of 5-AZA-dCTP into DNA produced a marked reduction in methyl acceptor ability mediated by DNA methylase.

TABLE 1

Summary of kinetic data for DNA polymerase reaction

K_m and K_i values are the means of at least three determinations ± standard deviation

Substrate	K_m	K_i	V_{max}
	μM	μM	*pmoles incorporated/ 20 min/mg protein*
dCTP	1.8 ± 0.8	2.2 ± 0.4	5500
5-AZA-dCTP	3.0 ± 0.7	4.3 ± 1.0	4630

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

TABLE 2

Template requirements of 5-AZA-dCTP incorporation

The reaction mixture (100 μl) contained 20 μmoles of potassium phosphate (pH 7.2), 0.6 μmole of $MgCl_2$, and 1.0 A_{260} of poly(dIC) or 0.5 A_{260} of poly(dAT). Where indicated, 10 nmoles of [^{3}H]dTTP (4.0×10^4 cpm) and 50 nmoles of dATP or dGTP and 0.03 unit of DNA polymerase α were added to the reaction mixture. Where indicated, also, either 10 nmoles of [^{3}H]dCTP (4.7×10^4 cpm) or 10 nmoles of [^{3}H]5-AZA-dCTP (1.2×10^4 cpm) and 50 nmoles of dATP or dGTP were added to the mixture. The reaction mixtures were incubated at 37° for 20 min.

Substrate	Template	Incorporation produced by DNA polymerase
		pmoles/20 min/mg protein
dTTP	poly(dIC)	<2
dCTP		23
5-AZA-dCTP		16
dTTP	poly(dAT)	107
dCTP		<2
5-AZA-dCTP		<2

The use of different concentrations of 5-AZA-dCTP in the above reaction showed that the greater the extent of incorporation of this nucleotide analogue into DNA, the greater the reduction of the template activity for DNA methylase. In a similar experiment using [^{3}H]5-AZA-dCTP, we estimated that as little as 1 nucleotide incorporated out of 2000 base residues reduced template activity by more than 50% (data not presented).

DISCUSSION

Since the lethal action of 5-AZA-dCyd is suspected to be due to its incorporation into DNA (2, 7), it was of interest to study the interactions of 5-AZA-dCTP with purified mammalian DNA polymerase. We also studied the effects of this incorporation on the methylation of cytosine in DNA in a reaction catalyzed by purified DNA methylase. Our results showed that the affinity of the catalytic site of DNA polymerase α for 5-AZA-dCTP was similar to that of dCTP as indicated by the similar K_m values for these substrates (Table 1). In addition, dCTP and 5-AZA-dCTP incorporation into DNA proceeded at a comparable rate as shown by their similar V_{max} values. These results demonstrate that the two substrates have the same potential to be incorporated into DNA. 5-AZA-dCTP and dCTP were also found to be weak competitive inhibitors of each other as indicated by their similar K_i values (Table 1). These results indicate that the catalytic site of the polymerase did not differentiate between these two substrates, suggesting that the molecular conformation of 5-AZA-dCTP is very similar to that of dCTP.

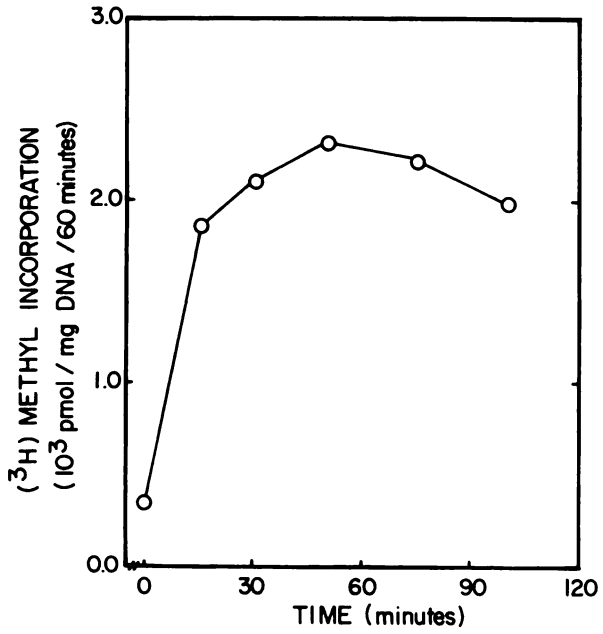

FIG. 3. *Effect of duration of the nick-translation reaction with 5-methyl-dCTP on the methyl acceptor activity of DNA*

Different incubation times were used to show the effect of nick-translation reaction containing 5-methyl-dCTP on template activity for DNA methylase.

TABLE 3

Incorporation of 5-AZA-dCTP into DNA inhibits DNA methylase

Hemimethylated DNA was prepared by using 5 methyl-dCTP. After the removal of 5-methyl-dCTP by spin dialysis, 5-AZA-dCTP or dCTP was incorporated into the hemimethylated DNA. The DNA was then used as a template for DNA methylase.

Experimental condition	Methyl incorporated	Inhibition
	pmoles	%
Control	22.9 ± 1.5	—
+5-AZA-dCTP, 4.25 μM	4.0 ± 1.6	84
+5-AZA-dCTP, 0.50 μM	7.5 ± 0.4	63
+5-dCTP, 4.25 μM	21.0 ± 0.2	8
+5-dCTP, 0.50 μM	22.6 ± 2.0	1

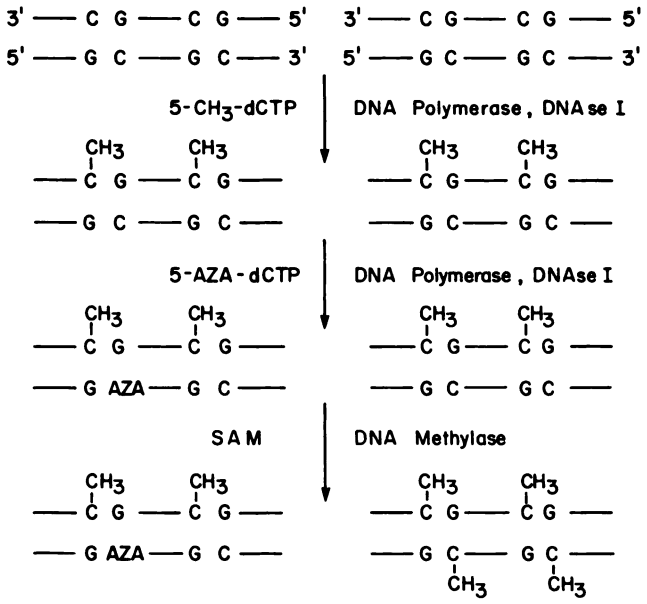

FIG. 4. *Experimental procedure used to prepare DNA template containing 5-AZA-cytosine residues*

The first step involved preparation of hemimethylated DNA with 5-methyl-dCTP (*5-CH_3-dCTP*). Second, 5-AZA-dCTP was incorporated into hemimethylated DNA. Third, the DNA containing 5-azacytosine (*AZA*) was tested for its ability to accept methyl groups from 5-adenosyl-L-methionine (*SAM*) in a reaction catalyzed by DNA methylase.

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

Data on template specificity suggest that the incorporation of 5-AZA-dCTP follows Watson-Crick rules of base pairing, since the nucleotide analogue was incorporated into poly(dIC) but not into poly(dAT) (Table 2). These template requirements are in agreement with quantum chemical calculations on the base pairing of 5-azacytosine with guanine (23). In support of our interpretation of these data is the report of Landolph and Jones (24), which show that 5-AZA-dCyd is not an active mutagen in mammalian cells. Our kinetic and template experiments indicate that 5-AZA-dCTP was as good a substrate as dCTP to be incorporated into DNA by mammalian DNA polymerase α and are in accord with cellular studies which show that radiolabeled 5-AZA-dCyd is readily incorporated into DNA of mammalian cells (2, 14).

The actual mechanism by which 5-AZA-dCyd produces its lethal effect as a result of its incorporation into DNA is not known. It is possible that the chemical instability of the azacytosine ring of this antimetabolite (8) may interefere with DNA function. Alternatively, the inhibition of DNA methylation observed in cells treated with 5-AZA-dCyd (9, 10, 14), may be an important factor relative to its antineoplastic activity, since DNA methylation appears to play an important role in gene expression and cellular differentiation (12). Therefore, it was important to investigate this phenomenon. 5-AZA-dCyd may act against leukemic cells by inducing them to differentiate so as to lose their proliferative potential (25). This hypothesis prompted us to analyze the consequences of 5-AZA-dCTP incorporation into DNA with respect to DNA methylation.

Our experimental approach was to incorporate 5-AZA-dCTP into DNA using DNA polymerase and to use this 5-azacytosine-containing DNA as a template for DNA methylase. Since hemimethylated DNA (presence of 5-methylcytosine in one strand of duplex DNA) is the best template for DNA methylase (22), we used the nick-translation reaction (18) to incorporate 5-methyl-dCTP into DNA. Under the experimental conditions used in this report, the optimal incubation time for this reaction was 50–60 min (Fig. 3). Longer incubations resulted in a significant reduction in template activity for DNA methylase, possibly due to the incorporation of 5-methyl-dCTP into both strands of DNA. A second-nick-translation reaction was used to incorporate 5-AZA-dCTP into the hemimethylated DNA.

The incorporation of 5-AZA-dCTP, but not dCTP, into hemimethylated DNA produced a significant inhibition of the DNA methylase reaction (Table 3). Very low levels of 5-AZA-dCyd incorporation markedly reduced the template activity of the DNA for DNA methylase. It was estimated that the presence of 1 5-azacytosine moiety out of 2000 base residues produces greater than 50% inhibition. Free 5-AZA-dCTP at a concentration of 8.5 μM did not directly inhibit DNA methylase (data not presented). This result shows that the incorporation of 5-AZA-dCTP into DNA inhibits DNA methylase activity. These results agree well with the observations that treatment of cells with 5-azacytosine analogues produces a significant inhibition of the methylation of cytosine residues in DNA (9, 10, 13, 26).

In cellular studies the inhibition of DNA methylation by 5-azacytosine analogues was clearly demonstrated by measuring the incorporation of radioactive uridine into 5-methylcytosine and cytosine in DNA (10, 13, 26, 27). Since the DNA from these cells was hypomethylated, it was a better template for DNA methylase than DNA from untreated cells. Apparently, in the intact cell the presence of 5-azacytosine in DNA blocks the progression of DNA methylase along the newly synthesized DNA, producing hemimethylated DNA, which is an excellent *in vitro* template for this enzyme (28). However, if there is extensive incorporation of the analogue into DNA so that 5-azacytosine is present at most of the potential methylation sites, there is a significant reduction in template activity (13, 27, 28, 29). In our study, starting with hemimethylated DNA, which is already a very active substrate for DNA methylase, we demonstrated that the incorporation of 5-AZA-dCTP into DNA produces an inhibition of this enzyme (Table 3).

The data presented in this report give some insight into the mechanism of action of the antineoplastic agent, 5-AZA-dCyd, with respect to its inhibition of DNA methylation in cells. The incorporation of 5-AZA-dCTP into specific nucleotide sequences of DNA may prove to be an interesting tool in studies on DNA-mediated transformation to elucidate further the biological role of DNA methylation.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Georges E. Rivard for his comments on this work and to Mrs. Sylvie Tassé for typing the manuscript.

REFERENCES

1. Šorm, F., and J. Veselý. Effect of 5-AZA-2′-deoxycytidine against leukemic and hemopoietic tissues in AKR mice. *Neoplasma (Bratisl.)* **15**:339–343 (1968).
2. Veselý, J., and A. Čihák. Incorporation of a potent antileukemic agent, 5-Aza-2′-deoxycytidine, into DNA of cells from leukemic mice. *Cancer Res.* **37**:3684–3689 (1977).
3. Momparler, R. L., and F. A. Gonzales. Effect of intravenous infusion of 5-Aza-2′-deoxycytidine on survival time of mice with L1210 leukemia. *Cancer Res.* **38**:2673–2678 (1978).
4. Rivard, G. E., R. L. Momparler, J. Demers, P. Benoit, R. Raymond, K. T. Lin, and L. F. Momparler. Phase I study on 5-AZA-2′-deoxycytidine in children with acute leukemia. *Leukemia Res.* **5**:453–462 (1981).
5. Momparler, R. L., and J. Goodman. In vitro cytotoxic and biochemical effects of 5-Aza-2′-deoxycytidine. *Cancer Res.* **37**:1636–1639 (1979).
6. Veselý, J., A. Čihák, and F. Šorm. Characteristics of mouse leukemic cells resistant to 5-azacytidine and 5-Aza-2′-deoxycytidine. *Cancer res.* **28**:1995–2000 (1968).
7. Momparler, R. L., J. Veselý, L. F. Momparler, and G. E. Rivard. Synergistic action of 5-Aza-2′-deoxycytidine and 3-deazauridine on L1210 leukemic cells and EMT_6 tumor cells. *Cancer Res.* **39**:3822–3827 (1979).
8. Lin, K. T., R. L. Momparler, and G. E. Rivard. High-performance liquid chromatographic analysis of chemical stability of 5-AZA-2′-deoxycytidine. *J. Pharm. Sci.* **70**:1228–1232 (1981).
9. Jones, P. A., and S. M. Taylor. Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **20**:85–93 (1980).
10. Creusot, P., G. Acs, and J. K. Christman. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-Aza-2′-deoxycytidine. *J. Biol. Chem.* **257**:2041–2048 (1982).
11. Jones, P. A., S. M. Taylor, T. Mohandas, and L. J. Shapiro. Cell cycle specific reactivation of an inactive X-chromosome locus by 5-azadeoxycytidine. *Proc. Natl. Acad Sci. U. S. A.* **79**:1215–1219 (1982).
12. Razin, A., and A. D. Riggs. DNA methylation and gene function. *Science (Wash. D. C.)* **210**:604–610 (1980).
13. Jones, P. A., and S. M. Taylor. Hemimethylated duplex DNAs prepared from 5-azacytidine treated cells. *Nucleic Acids Res.* **9**:2933–2947 (1981).
14. Adams, R. L. P., J. Fulton, and D. Kirk. The effect of 5-azadeoxycytidine on cell growth and DNA methylation. *Biochim. Biophys. Acta* **697**:286–294 (1982).
15. Momparler, R. L., M. Rossi, and A. Labitan. Partial purification and properties of two forms of deoxyribonucleic acid polymerase from calf thymus. *J.*

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

Biol. Chem. **248**:285–293 (1973).
16. Roy, P. H., and A. Weissbach. DNA methylase from Hela cell nuclei. *Nucleic Acids Res.* **2**:1669–1684 (1975).
17. Bollum, F. J. Filter paper disk techniques for assaying radioactive macromolecules. *Methods Enzymol* **12B**:169–173 (1968).
18. Rigby, P. W. J., M. Dreckmann, C. Rhodes, and P. Berg. Labeling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. *J. Mol. Biol.* **113**:237–251 (1977).
19. Momparler, R. L., and G. A. Fischer. Mammalian deoxyribonucleoside kinase. I. Deoxycytidine kinase: purification, properties, and kinetic studies with cytosine arabinoside. *J. Biol. Chem.* **243**:4298–4304 (1968).
20. Neal, M. W., and J. R. Florini. A rapid method for desalting small volumes of solution. *Anal. Biochem.* **55**:328–330 (1973).
21. Lineweaver, H., and D. Burk. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**:658–666 (1934).
22. Gruenbaum, Y., H. Cedar, and A. Razin. Substrate and sequence specificity of a eukaryotic DNA methylase. *Nature (Lond.)* **295**: 620–622 (1982).
23. Pithová, P., A. Píškala, J. Pitha, and F. Šorm. Nucleic acid components and their analogues. LVIII. 5-Azacytidine and related pairing with purine derivatives. *Collect. Czech. Chem. Commun.***30**:1626–1634 (1965).
24. Landolph, J. R., and P. A. Jones. Mutagenicity of 5-azacytidine and related nucleosides in C3H/10T½ clones 8 and V79 cells. *Cancer Res.* **42**:817–823 (1982).
25. Sachs, L. Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukaemia. *Nature (Lond.)* **274**:535–539 (1978).
26. Tanaka, M., H. Hibasami, J. Nagai, and T. Ikeda. Effect of 5-AZA-cytidine on DNA methylation in Ehrlich's ascites tumour cells. *Aust. J. Exp. Biol. Med. Sci.* **58**:391–396 (1980).
27. Momparler, R. L., V. L. Wilson, and P. A. Jones. Correlation of inhibition of DNA methylation by 5-aza-2′-deoxycytidine and its antileukemic activity. *Proc. 13th Int. Cancer Congr.*, 316 (1982).
28. Taylor, S. M., and P. A. Jones. Mechanism of action of eukaryotic DNA methyltransferase: Use of 5-azacytosine-containing DNA. *J. Mol. Biol.* **162**:679–692 (1982).
29. Friedman, S. The inhibition of DNA(cytosine-5)methylases by 5-azacytidine: The effect of azacytosine-containing DNA. *Mol. Pharmacol.* **19**:314–320 (1981).

Send reprint requests to: Dr. R. L. Momparler, Centre de Recherche Pédiatrique, Hôpital Ste-Justine, 3175 Chemin Ste-Catherine, Montréal, Québec, Canada H3T 1C5.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012