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Kinetic Interaction of 5-AZA-2′-Deoxycytidine-5′-Monophosphate and Its 5′-Triphosphate with Deoxycytidylate Deaminase

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SUMMARY

5-AZA-2′-deoxycytidine-5′-monophosphate (5-AZA-dCMP) was tested as a substrate, and 5-aza-2′-deoxycytidine-5′-triphosphate (5-AZA-dCTP) was tested as an allosteric effector of purified spleen dCMP deaminase. Graphic analysis of the velocity of deamination of 5-AZA-dCMP versus its concentration gave a hyperbolic curve in which the estimated apparent K_m was 0.1 mM. Since this curve was not sigmoidal and 5-AZA-dCMP at low concentrations stimulated the rate of deamination of the natural substrate, dCMP, it was proposed that the binding of 5-AZA-dCMP to the allosteric enzyme dCMP deaminase induced the *R* form. At substrate saturation, the rate of deamination of dCMP was 100-fold greater than that of 5-AZA-dCMP. dTTP inhibited the deamination of 5-AZA-dCMP with first-order kinetics. This inhibition was reversed by either 5-AZA-dCTP or dCTP. However, dCTP alone produced only a weak activation of the deamination of 5-AZA-dCMP in comparison to the potent activation when dCMP was the substrate. 5-AZA-dCTP was just as effective as dCTP for the allosteric activation of the deamination of dCMP. These results indicate that dCMP deaminase can play an important role in the metabolism 5-aza-2′-deoxycytidine nucleotides and may possibly modulate some of the pharmacological activity of this antimetabolite.

INTRODUCTION

5-AZA-CdR[2], an analogue of deoxycytidine, is an effective antileukemic agent (1–3). Like most nucleoside analogues, 5-AZA-CdR must be converted to a nucleotide in order to be an active agent. Cells deficient in deoxycytidine kinase, the enzyme that catalyzes the phosphorylation of 5-AZA-CdR (4), are resistant to the inhibitory effects of this analogue (5). After 5-AZA-dCMP is converted to 5-AZA-dCTP, it competes with dCTP for the catalytic site of DNA polymerase (6). During short exposures, 5-AZA-CdR does not produce an inhibition of DNA, RNA, or protein synthesis (7). The antineoplastic action of 5-AZA-CdR appears to be related to its incorporation into DNA (8), which results in an inhibition of methylation of cytosine residues in DNA to prevent the formation of 5-methylcystosine (9–11). The inhibition of DNA methylation produced by 5-AZA-CdR activates gene expression and induces cellular differentiation (10, 11).

It is important to understand the nucleotide metabolism of 5-AZA-CdR because changes in the intracellular pools of the naturally occurring deoxynucleotides may modulate the action of nucleoside analogues (8, 12). For example, cells with an increased intracellular pool of dCTP are resistant to the deoxycytidine analogue, cytosine arabinoside (13). Also, treatment of cells with thymidine produces an enhancement of the antineoplastic action of 5-AZA-CdR (14). Thymidine, after its conversion to dTTP, produces a decrease in the intracellular pool of dCTP (15) by feedback inhibition of ribonucleotide reductase (16). dTTP is also a feedback inhibitor of dCMP deaminase (17).

The enzyme dCMP deaminase plays an important role in deoxynucleotide metabolism by modulating the intracellular pool size of dCTP and dTTP (18). Cells deficient in this enzyme have a reduced pool of dTTP and an increased pool of dCTP (18). The pharmacological activity of deoxycytidine analogues such as cytosine arabinoside can be modified by dCMP deaminase because they may be substrates for this enzyme or their activity may be modulated by the pool size of dTTP or dCTP (19, 20). In addition, cytosine arabinoside-5′-triphosphate is an effective activator of dCMP deaminase (21–23).

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[2] The abbreviations used are: 5-AZA-CdR, 5-aza-2′-deoxycytidine; 5-AZA-dCMP, the 5′-monophosphate of 5-AZA-CdR; 5-AZA-dCTP, the 5′-triphosphate of 5-AZA-CdR.

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dCMP deaminase is an allosteric enzyme with a molecular weight of 120,000 (ref. 24), dCTP is the allosteric activator and dTTP is the allosteric inhibitor of this enzyme (17, 24). Several lines of evidence suggest that there are at least three interconverting conformational isomers of dCMP deaminase (19, 25, 26). The proposed isomers are the activated conformation, *R* form, the inhibited conformation, *T* form (27), and the enzyme without ligands (25). The binding of dCTP to the enzyme changes the conformation to the *R* form, whereas the binding of dTTP changes it to the *T* form (25).

Since dCMP deaminase may play an important role with respect to the modulation of the metabolism of 5-AZA-CdR nucleotides, we have investigated the interaction of 5-AZA-dCMP and 5-AZA-dCTP with this enzyme.

EXPERIMENTAL PROCEDURES

Materials. dCMP, dCTP, and dTTP were obtained from Sigma Chemical Company (St. Louis, Mo.) or P-L Biochemical Corporation (Milwaukee, Wisc.). 5-AZA-CdR was obtained from Chemapol Foreign Trade Company (Prague, Czechoslovakia). 5-AZA-dCMP and 5-AZA-dCTP were synthesized enzymatically from 5-AZA-CdR using purified deoxycytidine kinase (28), nucleoside monophosphate kinase, and nucleoside diphosphokinase (Boerhinger, Mannheim Corporation, Montreal) and purified by anion exchange chromatography as described previously (6). Homogeneous dCMP deaminase was prepared from donkey spleen by the previously described procedure (29), which was slightly modified to include a purification step using affinity chromatography (30). The specific activity of the purified dCMP deaminase was 920 units/mg. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the deamination of 1 μmole of dCMP per minute at 38°.

Enzyme assay. The reaction mixture (0.3 ml) contained 33 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 67 mM NaCl, the indicated concentration of substrate (dCMP or 5-AZA-dCMP), and 0.06–1.6 μg of purified spleen dCMP deaminase. The mixture was incubated at 37° in a 1-mm light path cuvette, and the rate of deamination was determined at 285 nm (dCMP) or 245 nm (5-AZA-dCMP) using a Cary 118 spectrophotometer. The initial velocity during the 1st min of the reaction was determined. For the conversion dCMP to dUMP, a ΔA_M at 285 nm of 3000 (10 mm light path) was used to calculate the amount of deamination. From the spectrum of 5-AZA-dCMP after complete deamination by 3.2 μg of dCMP deaminase at 25° the ΔA_M at 245 nm for this reaction was estimated to be 4500 (10-mm light path). The experimental error for the spectophotometric assay was not greater than 5%.

RESULTS

Kinetics of deamination of 5-AZA-dCMP. The effect of different concentrations of 5-AZA-dCMP on its rate of deamination by dCMP deaminase is shown in Fig. 1. The plot of the velocity of the reaction versus substrate concentration gave a hyperbolic curve. The estimated apparent K_m of 5-AZA-dCMP from the data plotted according to Lineweaver-Burk was 100 μM. When these data were plotted according the Hill Equation (data not shown), the estimated value for *n* was 1.0. Comparison of the rate of deamination of dCMP and 5-AZA-dCMP in the presence of 1.0 μM dCTP showed that the natural substrate is deaminated at a rate 100-fold greater than that of the nucleotide analogue (Table 2).

Effect of dCTP, dTTP, and 5-AZA-dCTP on the deamination of 5-AZA-dCMP. The effect of dCTP and dTTP on the rate of deamination of different concentrations of

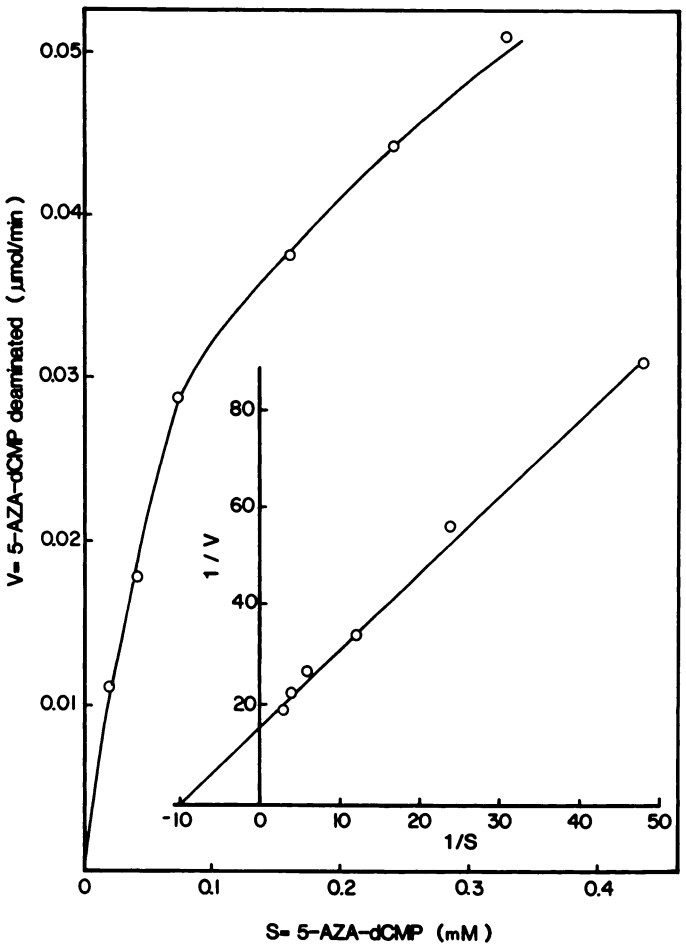

FIG. 1. *Effect of different concentrations of 5-AZA-dCMP on its rate of deamination*

The reaction mixture contained the indicated concentration of 5-AZA-dCMP and 1.6 μg of dCMP deaminase. The *inset* shows the data plotted according to the method of Lineweaver-Burk.

5-AZA-dCMP is shown in Fig. 2. At concentrations of 5-AZA-dCMP below 75 μM, dTTP at 30 μM inhibited the deamination of the nucleotide analogue by 100%. dCTP at 30 μM completely reversed this inhibitory effect of dTTP on the deamination of 5-AZA-dCMP. dCTP alone produced a slight, but significant, activation of the deamination of 5-AZA-dCMP at all substrate concentrations.

Figure 3 shows the effect of different concentrations of dTTP and dCTP on the rate of deamination of 98 μM 5-AZA-dCMP. dTTP produced 50% inhibition at a concentration of about 12 μM and >95% inhibition at 40 μM of the deamination of 5-AZA-dCMP. Maximal activation of the deamination of 5-AZA-dCMP was produced by 1.0 μM dCTP.

The effect of different concentrations of dCTP or 5-AZA-dCTP on the dTTP-induced inhibition of the deamination of 5-AZA-dCMP is shown in Table 1. dTTP at 30 μM completely inhibited the deamination of 41 μM 5-AZA-dCMP. dCTP at concentrations of 0.1, 0.3, and 1.0 μM reversed this dTTP-induced inhibition by 39, 89, and 100%, respectively. 5-AZA-dCTP was more effective than dCTP in reversing the inhibition induced by dTTP. At concentrations of 0.1 and 0.3 μM, 5-AZA-dCTP reversed this inhibition by 61 and 100%, respectively.

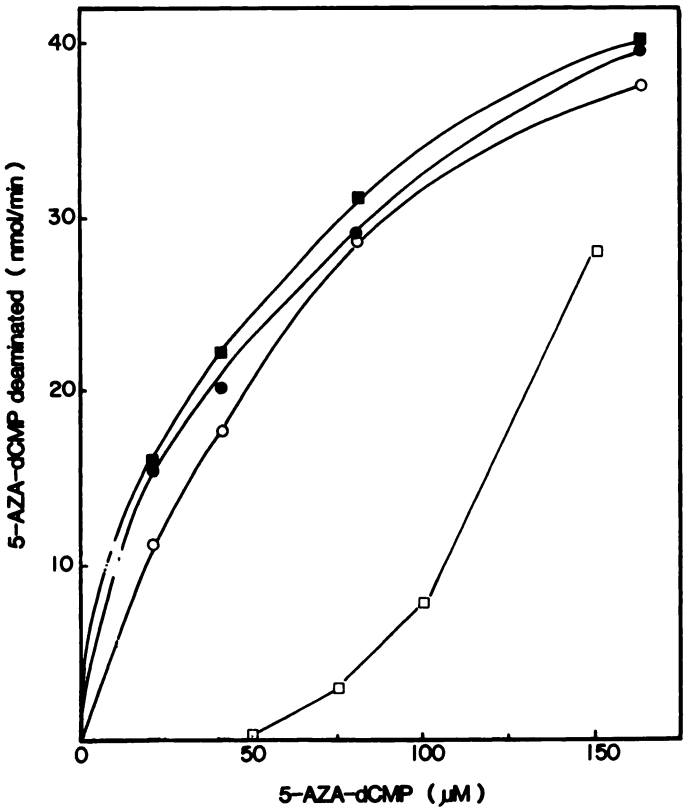

FIG. 2. *Effect of dCTP and dTTP on the rate of deamination of different concentrations of 5-AZA-dCMP*

The mixture contained 1.6 μg of dCMP deaminase and different concentrations of 5-AZA-dCMP alone (○) or with 30 μM dCTP (■), 30 μM dTTP (□), or 30 μM 5-AZA-dCTP + 30 μM dTTP (●).

Effect of dCTP, 5-AZA-dCTP, and 5-AZA-dCMP on the deamination of dCMP. The effect of different concentrations of 5-AZA-dCTP on the rate of deamination of dCMP is shown in Fig. 4. 5-AZA-dCTP was a potent activator of the deamination of the natural substrate. A concentration of 0.2 μM 5-AZA-dCTP produced a greater than 20-fold increase in the rate of deamination of dCMP.

A comparison of the different concentrations of dCTP or 5-AZA-dCTP which activate the deamination of dCMP is shown in Table 2. These data show that, at equimolar concentrations, 5-AZA-dCTP is just as effective as dCTP in activating the deamination of the natural

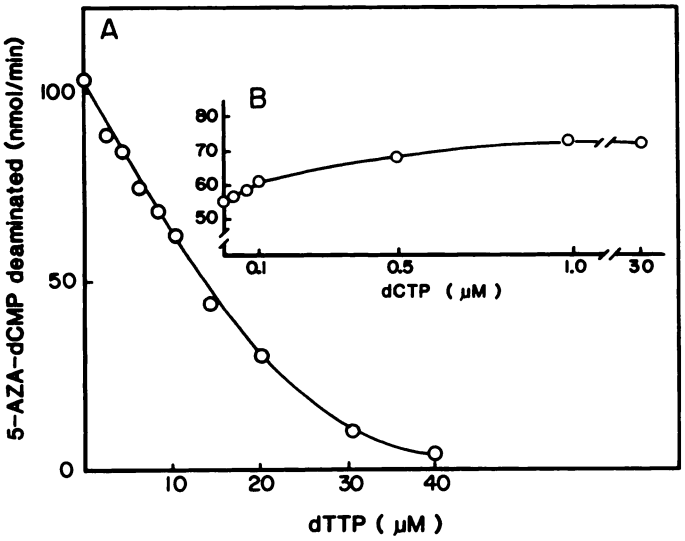

FIG. 3. *Effect of different concentrations of dTTP or dCTP on the rate of deamination of 5-AZA-dCMP*

In A, the reaction mixture contained 98 μM 5-AZA-dCMP; in B, it contained 49 μM 5-AZA-dCMP. Both reaction mixtures contained dCMP deaminase (5.3 μg/ml).

TABLE 1

Reversal of dTTP-induced inhibition of deamination of 5-AZA-dCMP by dCTP or 5-AZA-dCTP

The reaction mixture contained 41 μM 5-AZA-dCMP and dCMP deaminase (1.6 μg).

Additions			5-AZA-dCMP deaminated	Inhibition
dTTP	dCTP	5-AZA-dCTP		
	μM		*nmoles/min*	*%*
			18	0
30			<1	100
30	0.1		7	61
30	0.3		16	11
30	1.0		20	0
30		0.1	11	39
30		0.3	18	0
30		1.0	20	0

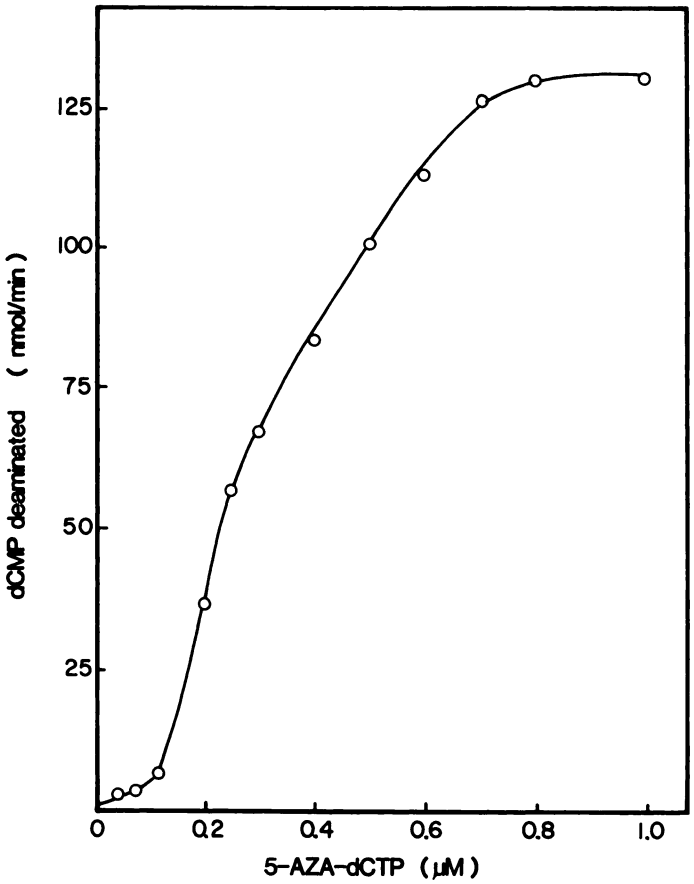

FIG. 4. *Effect of different concentrations of 5-AZA-dCTP on the rate of deamination of dCMP*

The reaction mixture contained 100 μM dCMP and dCMP deaminase (0.06 μg).

TABLE 2

Activation of the deamination of dCMP by dCTP or 5-AZA-dCTP

The reaction mixture contained 100 μM dCMP and dCMP deaminase (0.06 μg).

Addition	Concentration	dCMP deaminated
	μM	*nmoles/min*
None		<1
dCTP	0.2	27
dCTP	0.5	113
dCTP	1.0	123
5-AZA-dCTP	0.2	33
5-AZA-dCTP	0.5	103
5-AZA-dCTP	1.0	130

substrate, dCMP. Both dCTP and 5-AZA-dCTP at 0.2 μM produced >20-fold activation of deamination of dCMP.

The effect of different concentrations of 5-AZA-dCMP on the rate of deamination of dCMP is shown in Fig. 5. It was possible to determine the rate of deamination of dCMP in the presence of 5-AZA-dCMP, since this nucleotide analogue does not have any absorbance at 285 nm. At concentrations of 10 and 20 μM, 5-AZA-dCMP produced a marked activation of the rate of deamination of dCMP. Above 20 μM 5-AZA-dCMP, the extent of activation decreased with respect to increasing concentrations of the nucleotide analogue.

DISCUSSION

When 5-AZA-dCMP was used as the substrate for dCMP deaminase, the plot of the velocity of the reaction versus the substrate concentration gave a hyperbolic curve (Fig. 1). Analysis of these data by Hill plot (17) gave an n value of 1.0 for 5-AZA-dCMP. In general, for allosteric enzymes the plot of the velocity of the reaction versus the substrate concentration gives a sigmoidal curve. This is what was observed for dCMP deaminase when dCMP was the substrate (17). The sigmoidal curve indicates that there is a positive cooperative interaction between substrate molecules; that is, the binding of one molecule of dCMP to the enzyme enhances the binding affinity of the enzyme for the second molecule of dCMP. When such cooperativity exists, the n value from a Hill plot is greater than 1.0. For example, the n value for dCMP is 2.0 (17). For dCMP, the apparent K_m (substrate concentration at one-half the maximal velocity) is 0.49 mM (17). In the presence of the allosteric activator, dCTP, the enzyme is converted to the R form and the apparent K_m of dCMP decreases to 0.15 mM (17). The presence of a hyperbolic curve in the velocity versus substrate plot (Fig. 1) suggests that the binding of 5-AZA-dCMP to the enzyme converts it to the R form.

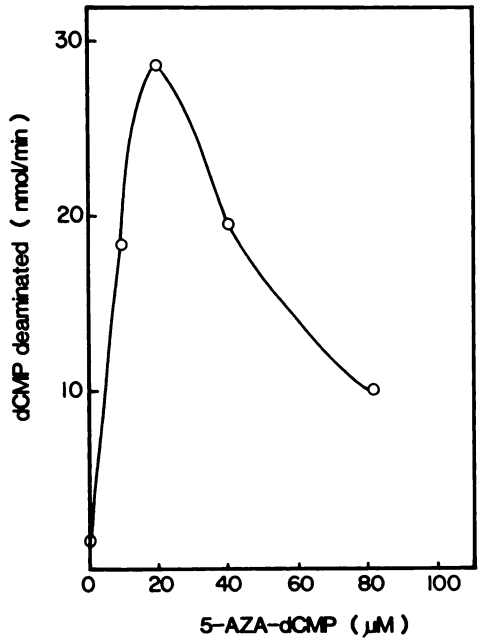

FIG. 5. *Effect of different concentrations of 5-AZA-dCMP on the rate of deamination of dCMP*

The reaction mixture contained 100 μM dCMP and dCMP deaminase (0.06 μg).

Although the apparent K_m of 5-AZA-dCMP (0.1 mM) and that of dCMP (0.15 mM in the presence of dCTP) were similar when the enzyme was in the R form, there was a marked difference in the rate of deamination of these two substrates. The natural substrate, dCMP, was deaminated at a rate that was about 100-fold greater than that of 5-AZA-dCMP. Since deamination takes place at position 4 of the cytosine ring, a change in the adjacent position 5 by substitution of a nitrogen for a carbon of the substrate can modify its interaction with the catalytic site of the enzyme.

Comparison of the kinetic data of dCMP with that of 5-AZA-dCMP for dCMP deaminase suggests that the binding of this substrate analogue to this enzyme changes its conformation to the R form. In support of this hypothesis are the data discussed above and the observation that low concentrations of 5-AZA-dCMP can activate the deamination of dCMP (Fig. 5). This latter effect has also been observed with dAMP, which at low concentrations activates the deamination of dCMP and at high concentrations acts as a competitive inhibitor (31). It has been proposed that dAMP induces the R form of the enzyme (31).

Since dCTP is an allosteric activator of dCMP deaminase (17), we investigated the effects of 5-AZA-dCTP on this enzyme. When dCMP was the substrate, 5-AZA-dCTP at equimolar concentrations was just as effective as dCTP as an allosteric activator of dCMP deaminase (Table 2). The effect of different concentrations of 5-AZA-dCTP on the rate of deamination gave a sigmoidal curve (Fig. 4), suggesting that a positive cooperativity between the allosteric effector sites occurred, as reported previously for dCTP (17). These data indicate that 5-AZA-dCTP is an allosteric activator of dCMP deaminase. In addition, 5-AZA-dCTP was as effective as dCTP in reversing the dTTP-induced inhibition of the deamination of 5-AZA-dCMP (Table 1). The inhibition of the deamination of 5-AZA-dCMP by different concentrations of dTTP did not appear to be of the cooperative type (Fig. 3), as observed previously when dCMP was the substrate (17).

One of the mechanisms by which thymidine can enhance the antileukemic action of 5-AZA-CdR (14) is by inhibition of the deamination of 5-AZA-dCMP by dTTP

in the reaction catalyzed by dCMP deaminase (Figs. 2 and 3; Table 1). However, as the intracellular pool of 5-AZA-dCTP increases, it can eventually block the inhibitory effect of dTTP on dCMP deaminase (Fig. 2; Table 1). Since the rate of deamination of dCMP is 100-fold greater than that of 5-AZA-dCMP, 5-AZA-dCTP activation of dCMP deaminase can potentially produce a "self-potentiation" of the over-all action of 5-AZA-CdR by reducing the competing deoxycytidine nucleotide pools, as proposed previously for cytosine arabinoside (21). In this regard, 5-AZA-dCTP is a much more potent activator of dCMP deaminase than the deoxycytidine nucleotide analogue, cytosine arabinoside-5′-triphosphate (21–23). In order to fully understand these effects, studies on intact cells should be performed.

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