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Adenosine analogs inhibit the guanine-7-methylation of mRNA cap structures

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The adenosine analogs neplanocin A and deazaneplanocin A were observed to inhibit the in vivo guanine-7-methylation of mRNA cap structure using a new assay for hypomethylated RNA. Treatment of cultured mammalian cells with these adenosine analogs resulted in the same extent of hypomethylation of cap structure as did ethionine injection in mice. Neplanocin A and its non-metabolizable analog 3-deazaneplanocin A show the same maximal level of inhibition of methylation suggesting that these adenosine analogs exert their effects by elevating *S*-adenosylhomocysteine levels rather than by conversion to other inhibitory compounds.

Guanine-7-methyltransferase; mRNA methylation; Cap; Ethionine; Neplanocin A; 3-Deazaneplanocin A

1. INTRODUCTION

Eucaryotic mRNAs contain a 5′-cap structure ($G^mpppX_mpN_mpN$---) which is highly modified via the methylation of both base and ribose components of its nucleotides [1]. This cap structure plays an important role in directing the splicing of the mRNA precursors [2] and in the initiation of translation [3,4]. Among the most important of these methylations is that of the 7-position of the guanine base, as the cap is not functional without it [2,5]. The roles of the other cap methylations and the methylation of internal bases such as N^6-methyladenosine are not clear, but may play a role in processing and/or transport to the cytosol [6].

Various adenosine analogs have been used to inhibit methylation of RNA by raising *S*-adenosylhomocysteine (SAH) levels through the inhibition of SAH hydrolase [7]. At elevated levels, SAH inhibits many methyltransferases resulting in the hypomethylation of mRNA and other RNAs as well. However, it has been reported that the adenosine analog Neplanocin A (NPC) does not inhibit the guanine-7-methylation of the cap structure even though it produced elevated SAH levels in the cell [8]. Similarly, the SAH analog *S*-tubercidinyl-homocysteine reportedly failed to inhibit the guanine methylation of the cap [6]. In both cases, while the 2′-*O*-methylation sites in the cap structure and internal N^6-methyladenosine sites were inhibited with these analogs, the important guanine-7-methylation was not affected.

We have partially purified the (guanine-7-)methyltransferase from mouse Ehrlich ascites cells [9] and have determined that it has a low K_i value (0.4 μM) for SAH (unpublished). Using this enzyme, an assay for determining the relative levels of unmethylated cap structure in mRNA (GpppXpN---) has been developed. Using this assay, we have examined the effects of two adenosine analogs, neplanocin A (NPC) and 3-deazaneplanosin A (deazaNPC) (see structures, Fig. 1), and the methionine analog ethionine on guanine-7-methylation of the cap structure.

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Abbreviations: SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SAE, *S*-adenosylethionine; NPC, neplanocin A; deazaNPC, 3-deazaneplanocin A.

2. MATERIALS AND METHODS

2.1. *Materials*

Chemically synthesized neplanocin A [10] and deazaneplanocin A [11] (see structures, Fig. 1) were kindly provided by Dr. Victor E. Marquez, National Cancer Institute. All other chemicals were reagent grade. Radioactive [*methyl*-^{3}H]-*S*-adenosylmethionine was purchased from Du Pont with a specific activity of 10–15 Ci/mmol.

2.2. *Guanine-7-methyltransferase*

The (guanine-7-)methyltransferase was prepared from nuclei isolated from Ehrlich ascites cells grown in ICR mice [9]. Ten grams of nuclei were isolated [12] and suspended in 60 ml of hypotonic buffer-I (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 1 mM DTE, 2 mM EDTA, and 10% (v/v) glycerol). The proteinase inhibitor, PMSF (60 mM, dissolved in acetone and stored at −20°C was added to a final concentration of 1 mM. The nuclei were disrupted by 10 sonication cycles (a Fisher Sonic Dismembrator-Model 300 with a microprobe was used); a 20-s sonication followed by 40 s of cooling in an ice slurry. The sonicated nuclear suspension was centrifuged at 100,000 × *g* for 2 h. The nuclear supernatant was adjusted to 0.2 M NaCl and forced through a DEAE-Sephadex column (0.7 × 5 cm) with a syringe. The guanine-7- methyltransferase that passed through the column was purified about 8- to 10-fold and the endogenous nucleic acids were removed. This enzyme preparation was dialyzed (2 × 500 ml) overnight against enzyme storage buffer (hypotonic buffer-I containing 50% glycerol) and stored at −20°C.

2.3. *Post-polysomal RNA*

Post-polysomal supernatant RNA was prepared by homogenizing tissue or cells in a hypotonic buffer-II (10 mM Tris-HCl, pH 7.9, 10

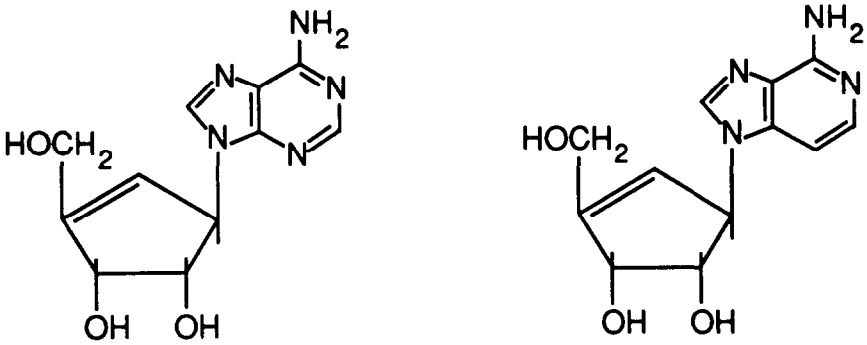

Fig. 1. Structures of adenosine analogs, neplanocin A (NPC) and 3-deazaneplanocin A (deazaNPC).

mM NaCl, 5 mM $MgCl_2$, and 1% Triton X-100) to break the cells. The homogenate was centrifuged at 1,000 × *g* for 10 min to pellet nuclei and cell debris and then at 20,000 × *g* for 20 min to remove mitochondria and lysosomes. The supernate was collected and centrifuged at 100,000 × *g* for 2 h to pellet polysomes. The post-polysomal fraction was mixed with 3 vols. of sodium dodecyl sulfate (SDS) buffer (50 mM sodium acetate, pH 5.1, 0.14 M NaCl, and 0.3% (w/v) SDS) and 4 vols. of phenol–cresol solution [13]. The resulting mixture was heated to 55°C with continuous stirring and cooled to room temperature on ice. The solution was stirred for an additional 30 min at room temperature and the phases were separated by centrifugation at 2,000 × *g* for 30 min. The aqueous phase was collected and the RNA was precipitated with 2.5 vols. of 95% ethanol (2% potassium acetate) at −20°C overnight. The RNA was collected by centrifugation and dissolved in autoclaved H_2O. The concentration was determined by measuring the absorbance at 260 nm ($E^{1\%}_{260nm}$=200).

2.4. *Tissue culture*

Normal rat kidney (NRK) cells were cultured at 37°C in Auto-Pow MEM (Flow Laboratories) supplemented with 5% fetal calf serum and 25 μg/ml gentamicin in an atmosphere of air/5% CO_2 [13]. When cultures at about passage 60 were 90% confluent, fresh media was added with various concentrations of adenosine analogs (NPC or deazaNPC). The cells were harvested after 24 h and the post-polysomal RNA was isolated as described above.

2.5. *Methyltransferase assay*

The methyltransferase assay contained 100 μl of the enzyme preparation, 1.4 μM [^{3}H]SAM (10–15 Ci/mmol), 0.05 M KCl, and 25 μg or 50 μg of post-polysomal RNA (the activity was proportional to RNA over the range from 10 μg to 200 μg) in a final volume of 150 μl. The assays were incubated at 37°C for 40 min (the reaction was linear for 60 min). The reactions were stopped by adding 200 μg of yeast tRNA as carrier in 0.5 ml of SDS buffer plus 0.5 ml of phenol–cresol to extract the RNA as described above. The RNA was repeatedly (four times) precipitated with ethanol and dissolved in H_2O to remove radioactive SAM. To determine total methyltransferase activity, the RNA was precipitated again with ethanol and the pellet was dissolved in 0.5 ml of H_2O and the total radioactivity was determined by liquid scintillation counting. The isolated RNA was dissolved in 500 μl of freshly prepared 0.3 N NaOH and incubated at 37°C for 16 h to hydrolyze the RNA (the cap structure is resistant to hydrolysis) in order to determine the type of methylation. The digested sample was diluted with 45 ml of urea buffer (25 mM Tris-HCl, pH 7.5 and 7 M urea) and applied to a DEAE-Sephadex column (0.7 × 20 cm) that had been equilibrated with urea buffer at a flow rate of about 1 ml/min. The column was washed with 10 ml of urea buffer and the oligonucleotides were eluted with a 200 ml linear salt gradient (0–0.4 M NaCl in urea buffer) [14]. Three-milliliter fractions were collected and the UV absorbance at 260 nm and radioactivity of each fraction were determined. Cap structures were eluted at about mid-point of the salt-gradient. The peaks of radioactivity eluting from the column correspond to the amount of in vivo hypomethylation in that RNA fraction, i.e. high radioactivity in the cap structures is proportional to a low level of methylation of these structures in the cell as a result of treatment with an analog.

2.6. *Ethionine treatment*

Mice (ICR mice 6–7 weeks old, about 30–35 g) were injected intraperitoneally with L-ethionine (125 mg/kg body weight) and adenine (120 mg/kg body weight) in 1 ml of sterile saline [9,15]. These injections were repeated twice at 24 h intervals and then the animals were sacrificed after another 24 h. The livers were removed and washed in cold saline and the post-polysomal RNA (methyl-deficient) was isolated.

3. RESULTS

The relative levels of unmodified methyl-acceptor groups in various post-polysomal RNA preparations were determined by incubating RNA with nuclear methyltransferase. The total levels of methyl-acceptor groups was determined in NRK cells treated with different concentrations of adenosine analogs, NPC and deazaNPC (Fig. 2). Both analogs had a IC_{50} of about 0.5 μM and maximally inhibited methylation at 10 μM NPC or deazaNPC in the culture media. Cytotoxic effects were observed with cells treated with 100 μM amounts of the adenosine analogs.

Fig. 3A shows the DEAE-Sephadex column profiles of alkaline digested mouse liver post-polysomal RNA from control and ethionine-treated mice after incubation with crude methyltransferase. The control RNA was a poor substrate for the methyltransferase while the RNA isolated from ethionine treated mice was an excellent methyl-acceptor with a double peak (fractions 20–35) representing cap structures with net charges of −5 (cap 0, G^mpppNp) and −6 (cap 1, G^mpppN_mpNp) and a small shoulder with a charge of −7 (cap 2, $G^mpppN_mpN_mpNp$). There was a very small peak around fraction 3 with a charge of −2 (mononucleo-

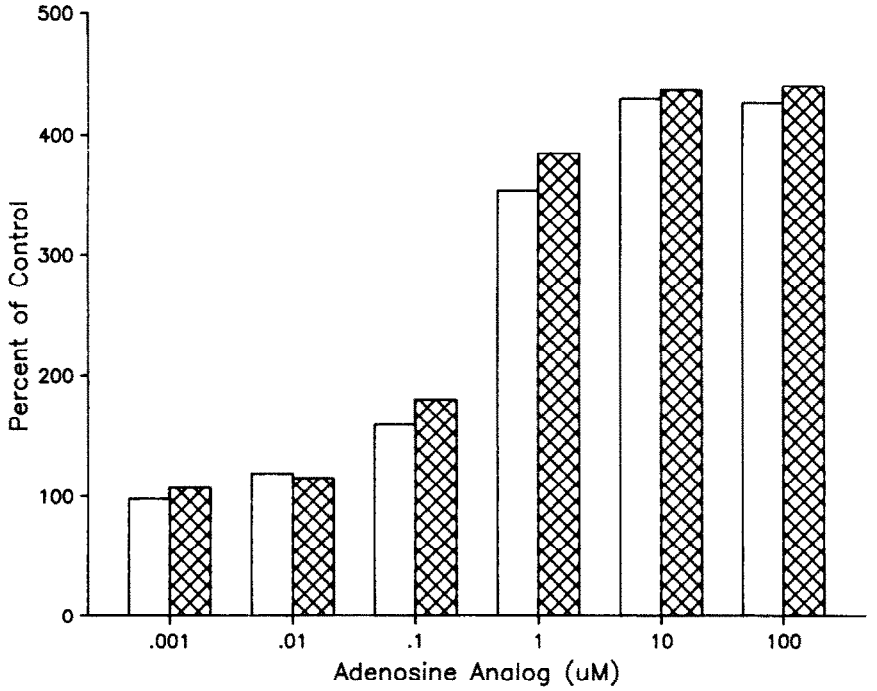

Fig. 2. Total methyl-acceptor levels in post-polysomal RNA isolated from NRK cells treated with various levels of adenosine analogs (NPC, open bars and deazaNPC, hatched bars). RNA samples (25 μg) isolated from methyltransferase assays were precipitated with ethanol (5×) to remove [^{3}H]SAM and the total radioactivity of each sample was determined. The control value (no adenosine analog) was 798 cpm (100%) and was the average of two flasks of cells.

tides) resulting from the base methylation of nucleotides internal in the RNA sequence.

Fig. 3B shows the DEAE-Sephadex column profiles of alkaline digested RNA from control and NPC-treated NRK cells. Cap 1 and cap 2 structures are the most prevalent with NPC treatment while ethionine treatment in mice produces mostly cap 0 and cap 1 structures (Fig. 3A and B). NPC treatment also generates many base hypomethylated sites on nucleotides internal in tRNA (fraction 5, Fig. 3B). While hypomethylation sites are low in control NRK cells (Figs. 2 and 3B) the levels are higher than seen in liver, consistent with hypomethylation levels previously observed with these tissue culture cells at this passage level [13].

The experiment was repeated using NPC treated cells and deazaNPC treated cells. NRK cells treated with deazaNPC, an adenosine analog that is not metabolized to its SAM analog, had similar levels of hypomethylation as cells treated with NPC (Fig. 4). Again, a large peak of methylated nucleotides (N^mp) was observed with both of these analogs.

4. DISCUSSION

We have previously used ethionine treatment of mice to produce methyl-deficient RNA (hypomethylated RNA) as a substrate for nuclear methyltransferases including the guanine-7-methyltransferase that methylates the cap structure [9]. Ethionine inhibits methylation by its metabolism to the SAM analog *S*-adenosylethionine (SAE), which acts as a substrate analog for many methyltransferases causing an inhibition of methylation [15]. Another inhibitor used in these experiments, NPC, is an adenosine analog (Fig. 1) that can inhibit the SAH-hydrolase [7] (metabolizes SAH to adenosine and homocysteine) resulting in a dramatic increase in cellular SAH levels which can inhibit many methyltransferases. NPC can also be metabolized to *S*-neplanosylmethionine (SAM analog) and to *S*-neplanosylhomocysteine (SAH analog) which could also cause inhibition of methylation [7]. Another adenosine analog, deazaNPC, is not metabolized to its SAM and SAH analog and only elevates SAH levels by the inhibition of SAH-hydrolase [11].

The multiple peaks of radioactivity that are resolved by column chromatography of digests of postpolysomal RNA correspond to the cap structures cap 0 (−5 charge, G^mpppNp), cap 1 (−6 charge, G^mpppN_mpNp) and cap 2 (−7 charge, $G^mpppNmpNmpNp$). The cap structures are resistant to alkaline digestion; however, the imidazole ring of guanine is opened at the 8–9 bond, converting 7-methylguanine to the ring-opened derivative, which lacks the positive charge [1]. Ethionine treatment generates RNA with more of the unmethylated cap 0 structure than the adenosine analogs because of the apparent inhibition by SAE of the 2′-*O*-methyltransferase that methylates the ribose in the nucleotide in the +1 position of the RNA (Figs. 3 and 4). The 2′-*O*-

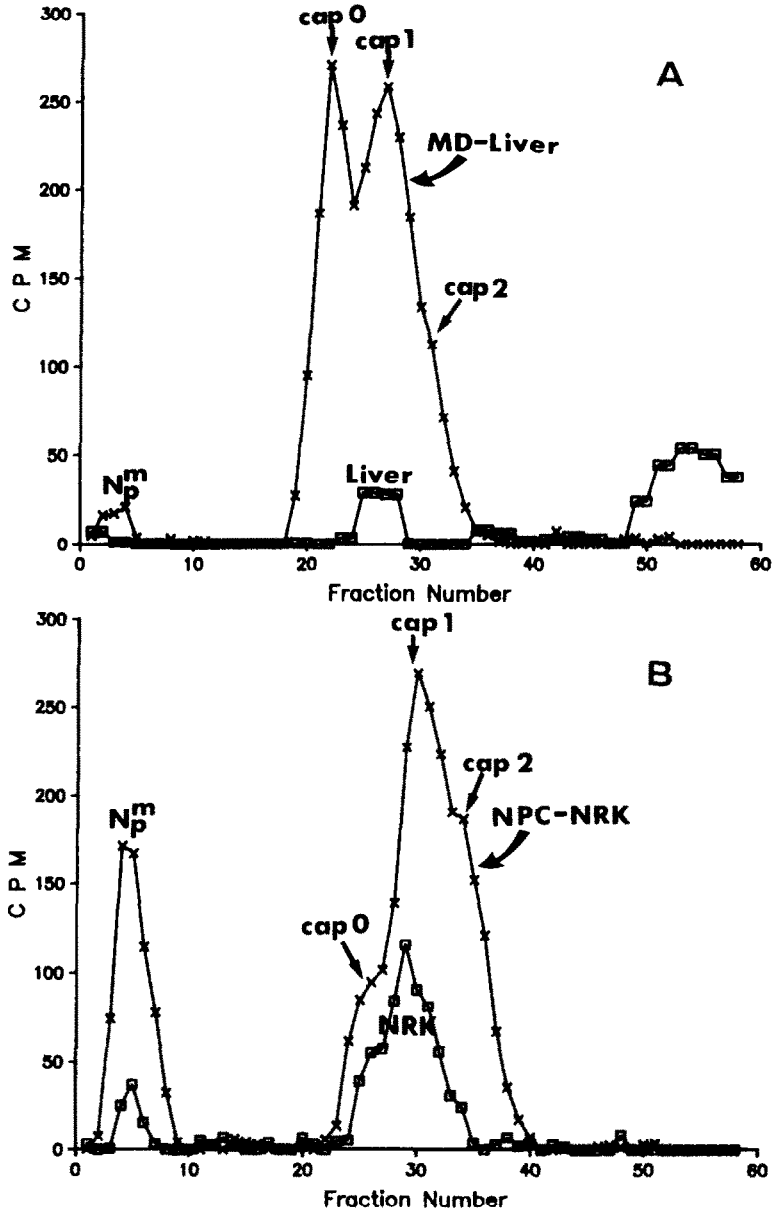

Fig. 3. Analysis of hydrolyzed-methylated RNA on DEAE-Sephadex columns. RNA (50 μg) isolated from a methyltransferase assay was digested with 0.3 N NaOH and applied to DEAE-Sephadex columns and nucleotides and oligonucleotides were eluted with a 0–0.4 M NaCl gradient (the gradient started at fraction 1 and ended at fraction 58). Panel A shows the data from mouse liver post-polysomal RNA (liver, □) and ethionine treated mouse liver post-polysomal RNA (MD-liver, ×) used as substrate for the methyltransferase. Panel B shows data from normal rat kidney post-polysomal RNA (NRK, □) and neplanocin A treated NRK post-polysomal RNA (NPC-NRK, ×). The position of the nucleotide peak is marked Np^m (−2 charge) while the cap structures are marked: cap 0 (−5 charge), cap 1 (−6 charge) and cap 2 (−7 charge).

methyltransferase that methylates the nucleotide in +1 position of the RNA is apparently more sensitive to elevated SAE than to high SAH levels. However, the adenosine analogs generate more unmethylated sites internal in the RNA sequences (N^mp peak seen in Figs. 3b and 4, predominantly due to hypomethylated tRNA which is the most prevalent RNA in the post-polysomal fraction) than ethionine treatment.

Since both adenosine analogs, NPC and deazaNPC, inhibit methylation to the same extent (Figs. 2 and 4), the inhibition of guanine-7-methylation of the cap structure must be primarily due to the elevation of SAH. We have observed a low K_i value (0.4 μM) for the inhibition of the isolated guanine-7-methyltransferase by SAH (unpublished). Because of the report that deazaNPC was a more potent inhibitor of the SAH hydrolase than NPC [16], we were surprised to find it caused identical maximal inhibition at 10 μM concentration (Figs. 2 and 4). However, a more recent report has established that deazaNPC inhibits SAH hydrolase at about the same level as NPC [17], which agrees with our data.

The earlier reports of the inability of NPC and *S*-

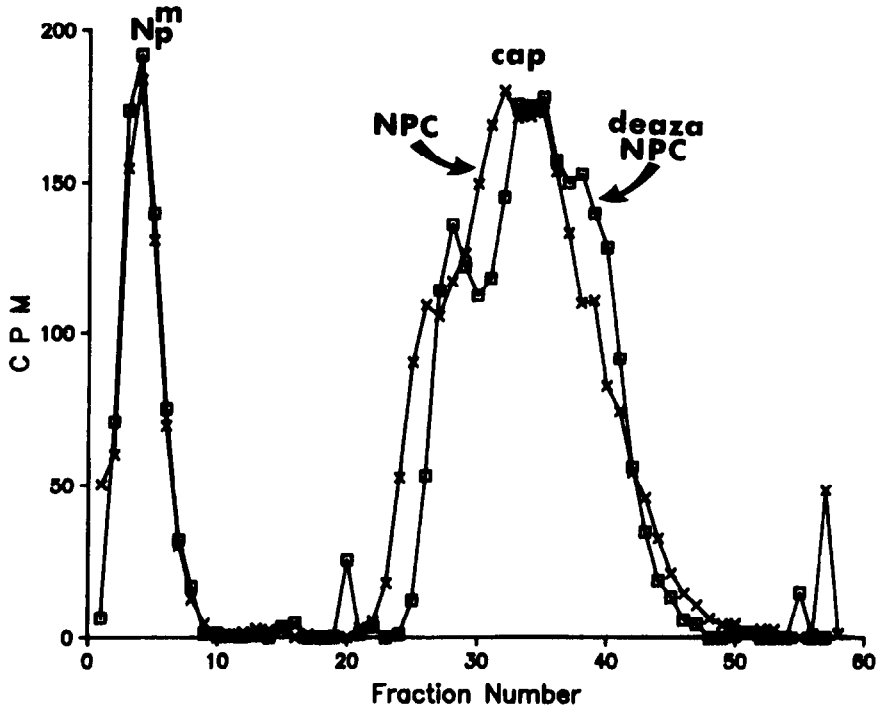

Fig. 4. DEAE-Sephadex column analysis of methylated RNA. Two samples of post-polysomal RNA isolated from NRK cells treated with 10μM neplanocin A (NPC, ×) or deazaneplanocin A (deazaNPC, □) were used as substrate for the nuclear methyltransferase and digested and analyzed as in Fig. 3 except that a 0–0.3 M NaCl gradient was used. The position of the nucleotide and cap peaks are marked Np^m and cap respectively.

tubercidinyl homocysteine to inhibit the guanine-7-methylation in cap structure [6,8], may have been due to the analysis of poly A selected RNA. We have found that if the mRNA is not guanine-7-methylated it does not bind to ribosomes, i.e. non-guanine-7-methylated capped mRNA appears in the post-polysomal fraction and is rapidly degraded to fragments about 40 to 60 nucleotides in size [9]. In this same study [9], we also found little unmethylated cap structure in the nuclear-RNA or polysomal-RNA, which is not surprising, as methylation in the 7-position of the guanine in the cap structure is required for ribosome binding [5].

The treatment of NRK cells with adenosine analogs resulted in similar levels of hypomethylation of cap structure as found in ethionine injection of mice. Therefore, the methylation of the guanine-7-methylation of cap structure in mRNA is sensitive to elevated SAH. We have found the use of the guanine-7-methyltransferase assay to be a sensitive method to detect unmethylated cap structures.

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