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Effect of Neplanocin A on Differentiation, Nucleic Acid Methylation, and c-*myc* mRNA Expression in Human Promyelocytic Leukemia Cells

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SUMMARY

The effect of the cyclopentenyl adenosine analog neplanocin A (NPC) on cell growth and differentiation was examined in the human promyelocytic leukemia cell line HL-60. Continuous exposure of HL-60 cells to 0.1–3.3 μM NPC resulted in a progressive reduction in cell growth which was accompanied by an increase in differentiation to cells with a myelocyte and neutrophil morphology. The latter effect was expressed as an increase in the capacity of cells to reduce nitro blue tetrazolium and reached a level of 40% of the total cell population. Preceding the phenotypic changes was the preferential inhibition of RNA and DNA methylation in comparison to inhibition of their synthesis which coincided with the formation of a metabolite of NPC with the chromatographic characteristics of *S*-adenosyl-L-methionine (AdoMet). In addition, c-*myc* mRNA expression, which is amplified in HL-60 cells, was markedly reduced following NPC treatment. These results indicate that NPC is an effective inhibitor of RNA and DNA methylation resulting from its conversion to an analog of AdoMet, and that these effects appear to be responsible for reduced c-*myc* RNA expression and the induction of myeloid differentiation in this cell line.

INTRODUCTION

NPC[1] is a cyclopentenyl analog of adenosine that was originally isolated from the soil fungus *Ampullariella regularis* (1). This antibiotic possesses significant antitumor activity against murine L1210 leukemia (1–3), shows moderate cytocidal activity against human colon carcinoma cells *in vitro* (4), and inhibits vaccinia virus multiplication in mouse L cells (5). Recently, the total synthesis of NPC was reported (6, 7) and the availability of this drug (6) has now allowed us to further evaluate its mechanism of action and antiproliferative activity.

In this report, we have assessed the growth inhibitory and differentiating properties of NPC against the human promyelocytic leukemia cell line HL-60. This cell line is unique in that it undergoes morphologic change to cells with the characteristics of mature myelocytes in response to a variety of differentiating agents (8). In addition, the cell line contains the amplified cellular oncogene, c-*myc* (9–11) which allows us to measure the regulation of a specific mRNA in response to NPC, a drug which appears to have a selective action on RNA methylation (4).

[1] The abbreviations used are: NPC, neplanocin A; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NBT, nitro blue tetrazolium; HPLC, high performance liquid chromatography; dCF, 2′-deoxycoformycin; SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0; AdoMet, *S*-adenosyl-L-methionine; SDS, sodium dodecyl sulfate; AdoHcy, *S*-adenosylhomocysteine; NPC-Met, AdoMet analog of NPC; NPC-Hcy, AdoHcy analog of NPC; m^{5}dCyd, 5-methyl-2′-deoxycytidine.

EXPERIMENTAL PROCEDURES

Materials. [U-^{14}C]Urd (506 mCi/mmol), [*methyl*-^{14}C]dThd (50.5 mCi/mmol), and [*methyl*-^{3}H]methionine (12 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA). dCF was provided by the Natural Products Branch, National Cancer Institute. NPC (6) was provided by Drs. Mu-Ill Lim and Victor E. Marquez, Laboratory of Medicinal Chemistry and Pharmacology, National Cancer Institute. The v-*myc* DNA probe (2–3 × 10^8 dpm/μg) was obtained from Oncor, Inc. (Gaithersburg, MD).

Tissue culture. HL-60 cells were obtained from Dr. Theodore R. Breitman, National Cancer Institute and were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 40 mM Hepes (pH 7.4), and 50 μg/ml gentamicin under an atmosphere of air:5% CO_2. The initial cell density was 5 × 10^5/10 ml of medium in 25-cm^2 flasks.

NBT assay. Cells were analyzed for their ability to reduce NBT to formazan as described by Breitman *et al.* (12).

RNA synthesis and methylation. Cells (5 × 10^5/10 ml of medium) were labeled with 50 μCi of [^{3}H]methionine (50 mCi/mmol) and 0.5 μCi of [^{14}C]Urd (506 mCi/mmol) concurrently with drug treatment for 24 hr, and RNA was isolated by the procedure described previously (4).

RNA was separated by electrophoresis in composite gels (0.5 × 11 cm) containing 1.9% acrylamide, 0.6% agarose, 40 mM Tris-HCl (pH 7.6), 20 mM sodium acetate, 3 mM EDTA, and 10% glycerol (4).

DNA synthesis and methylation. Cells (1.5 × 10^6/30 ml of medium) were labeled with 30 μCi of [^{3}H]methionine (10 mCi/mmol) and 1 μCi

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of [^{14}C]dThd (50.5 mCi/mmol) concurrently with drug treatment for 24 hr, and DNA was isolated and digested to deoxyribonucleosides as described previously (11). [^{3}H]m^{5}dCyd and [^{14}C]dThd in the DNA digests were separated and quantitated by reverse phase HPLC (13).

RNase T_2 digestion. RNA was isolated from cells labeled with [^{3}H] methionine and [^{14}C]Urd as described above and digested overnight with 100 units of RNase T_2 (4).

Metabolism of NPC. Cells were treated with varying concentrations of NPC for 1–3 days. Following treatment, 5% trichloroacetic acid extracts were prepared and the AdoMet-like metabolite of NPC was separated by reverse phase HPLC following incubation in the presence and absence of bacterial alkaline phosphatase (4).

Measurement of c-myc mRNA. c-*myc* RNA was measured by the "quick-blot" procedure of Bresser *et al.* (14). 4×10^6 cells were collected 24 and 48 hr after drug treatment, washed with 10 ml of phosphate-buffered saline (6.3 mM Na_2HPO_4, 0.8 mM KH_2PO_4, 0.154 M NaCl/500 μM cycloheximide, and resuspended in 0.5 ml of phosphate-buffered saline/500 μM cycloheximide/10 mM vanadyl riboside/0.2 mg/ml proteinase K, and 1/20 volume of 10% Brij-35 was added during the final 20 min of a 40-min incubation at 37°. One-twentieth volume of 10% sodium deoxycholate was then added and the sample was mixed and placed on ice for 5 min. Finally, 0.833 volume of supersaturated sodium iodide (2.5 g/ml) was added and mixed. After 5 min, appropriate dilutions were made into saturated sodium iodide (1.1 g/ml). Aliquots of the solutions were filtered onto nitrocellulose paper (*m*RNC, S+S, Keene, NH) which had been moistened with H_2O and soaked for longer than 5 min in 6× SSC. After filtration, the filter was soaked 3 times for 5 min each in H_2O, 3 times for 5 min each in 70% ethanol, and 10 min in 0.25% (v/v) acetic anhydride/0.1 M triethanolamine. Filters were prehybridized for 16–18 hr at 37° in 50% formamide, 0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidinone, 5× SSC, 50 mM Na_2HPO_4 (pH 6.5), 100 μg/ml sonicated denatured salmon sperm DNA, and 0.1% SDS. Hybridization was for 16–18 hr at 37° in the above solution made 10% in dextran sulfate and containing approximately 10^6 dpm of v-*myc* [^{32}P]DNA probe/ml. After hybridization, the filter was washed 3 times with 1× SSC/0.1% SDS for 5 min at room temperature and 3 times with the same buffer for 30 min at 50°. Filters were dried and autoradiographed on Kodak X-Omat XAR-2 film with two intensifying screens. mRNA levels were quantitated by densitometry of the autoradiographs (Hoefer Scientific, GS 300 scanning densitometer).

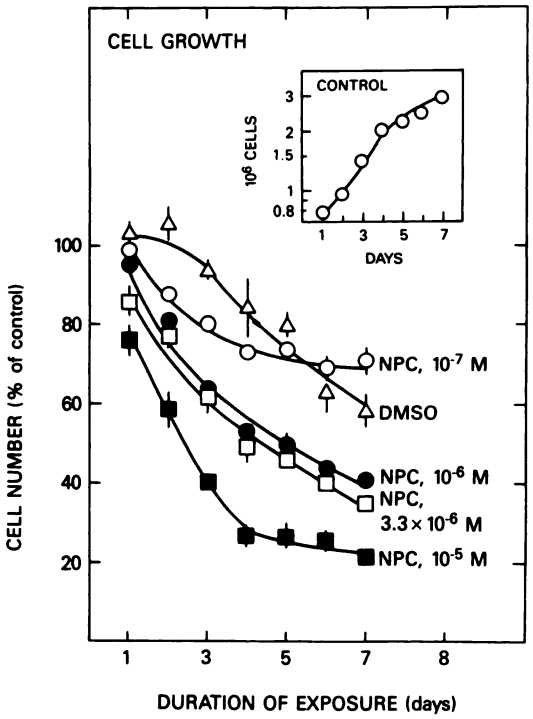

FIG. 1. *Cell growth following NPC treatment*
Cells were exposed continuously to varying concentrations of NPC or 1.6% (v/v) DMSO. Inset, growth rate of control cells. Each value is the mean ± standard error of four to five experiments.

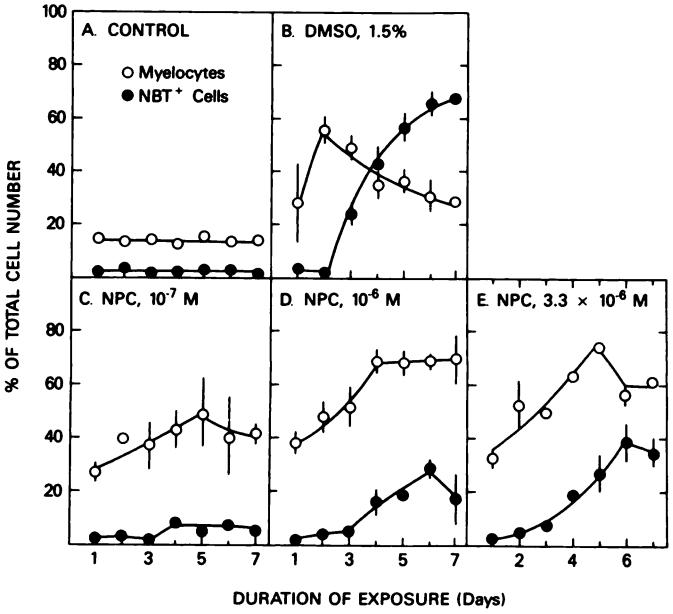

FIG. 2. *Differentiation of HL-60 cells following NPC treatment*
Cells were exposed continuously to varying concentrations of NPC or 1.6% (v/v) DMSO and the number of myelocytes or NBT-positive cells was determined. Each value is the mean ± standard error of three to four experiments.

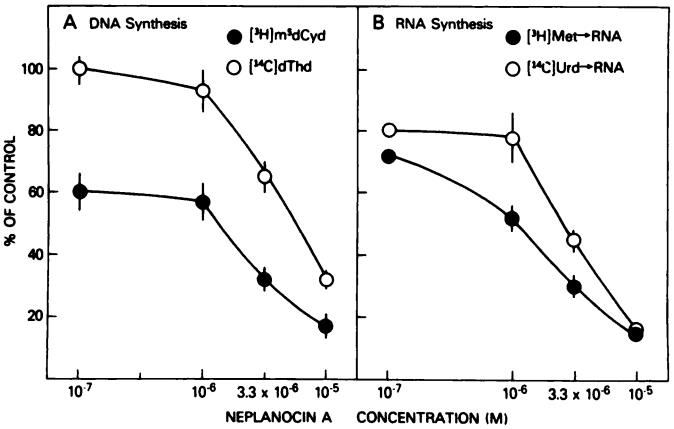

FIG. 3. *Methylation and synthesis of DNA and RNA following NPC treatment*
Cells were exposed for 24 hr to varying concentrations of NPC and with [^{3}H]methionine and [^{14}C]dThd to label DNA (A) or [^{3}H]methionine and [^{14}C]Urd to label RNA (B). DNA and RNA were extracted as described under Experimental Procedures. Each value is the mean ± standard error of five experiments.

RESULTS

Cell growth and differentiation. The growth of HL-60 cells following continuous exposure to NPC is depicted in Fig. 1. NPC at 0.1 μM inhibited cell growth by 30%, while 1 to 3.3 μM NPC inhibited cell growth by 60–65% after 7 days. Ten μM NPC was markedly inhibitory,

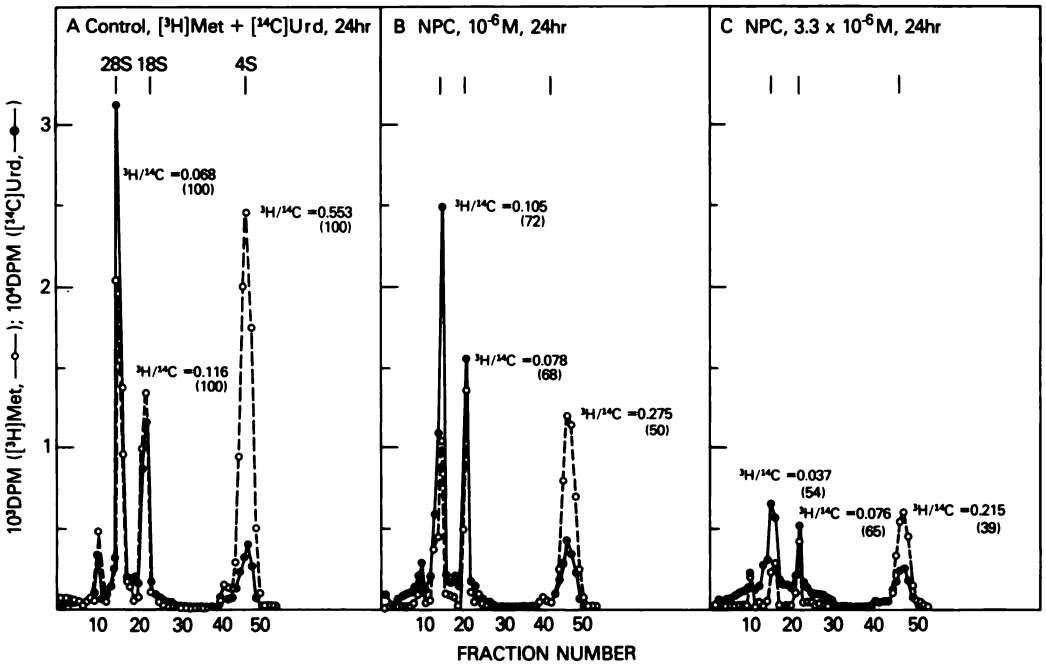

FIG. 4. *Methylation and synthesis of rRNA and tRNA following treatment with NPC*

Cells were exposed for 24 hr to NPC and [^{3}H]methionine and [^{14}C]Urd and RNA was isolated and separated by gel electrophoresis as described under Experimental Procedures. Numbers in parentheses indicate the per cent change in the ratio of ^{3}H/^{14}C versus control RNA.

TABLE 1

Methylation and synthesis of RNA following NPC treatment

Cells were treated with NPC and [^{3}H]methionine and [^{14}C]Urd as described in Fig. 4. Each value is the mean of two experiments.

Drug treatment	RNA fraction					
	28 S		18 S		4 S	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
	dpm					
Control	3,100	39,000	3,500	17,600	7,900	13,600
	% control					
NPC, 1 μM	50	76	50	104	54	107
NPC, 3.3 μM	19	34	22	44	31	74

reaching a maximum effect in 4 days. Simultaneous measurements of NBT-positive cells that are indicative of mature neutrophils, as well as myelocytes by morphologic criteria, revealed that NPC markedly increased myeloid differentiation (Fig. 2). Increased differentiation of HL-60 cells to myelocytes appeared as early as 1 day after treatment while the appearance of NBT-positive cells required a minimum of 4 days, and reached a maximum of 40% of the cell population by day 6. The response of cells to NPC also differed from that of the myeloid differentiating agent, DMSO, where the appearance of NBT-positive cells increased markedly after 3 days and reached 70% of the cell population within 7 days concomitantly with a reduction in the number of myelocytes.

DNA and RNA methylation. Cells were incubated for 24 hr concurrently with NPC and either [^{3}H]methionine and [^{14}C]dThd to label DNA (Fig. 3A) or [^{3}H]methionine and [^{14}C]Urd to label RNA (Fig. 3B). NPC produced a preferential reduction in the methylation of DNA, and to a lesser extent of RNA versus inhibition of their synthesis at concentrations which produced both growth inhibition and myeloid differentiation.

Further analysis of cellular RNA by electrophoresis revealed a greater differential effect on the methylation of tRNA (4 S RNA) versus rRNA (28 S + 18 S RNA) (Fig. 4 and Table 1). The ratio of ^{3}H/^{14}C incorporated from [*methyl*-^{3}H]methionine and [^{14}C]Urd indicated a 30–55% preferential reduction in the methylation of rRNA and 50–60% reduction in the methylation of tRNA by 1–3.3 μM NPC (Fig. 1). The synthesis and methylation of tRNA were reduced by 26 and 69%, respectively, at 3.3 μM NPC, whereas 28 S + 18 S rRNA synthesis was inhibited by 66–69% and methylation by 80% at the same concentration of NPC (Table 1).

To determine whether NPC produced a differential effect on base methylation or 2′-*O*-methylation of ribose, RNA was digested completely with RNase T_2 and the mononucleotides ("Np") and dinucleotides ("NpNp") generated were separated by their net charge differences by anion exchange HPLC (Fig. 5 and Table 2). NPC inhibited methylation in both the mononucleotide fraction (base methylation) and dinucleotide fraction (2′-*O*-methylation) equally (Table 2). However, examination of the chromatogram indicated that specific and as yet unidentified methylated bases in RNA are highly sensitive to the inhibitory effect of NPC (Fig. 5, arrow).

c-myc mRNA levels. HL-60 cells contain the amplified cellular oncogene, c-*myc* (8–10). To determine whether the amplified expression of c-*myc* mRNA was affected by NPC treatment, dot blot analysis was performed using a v-*myc* DNA probe (Fig. 6). The level of c-*myc* mRNA declined following exposure of cells to NPC for 1 or 2

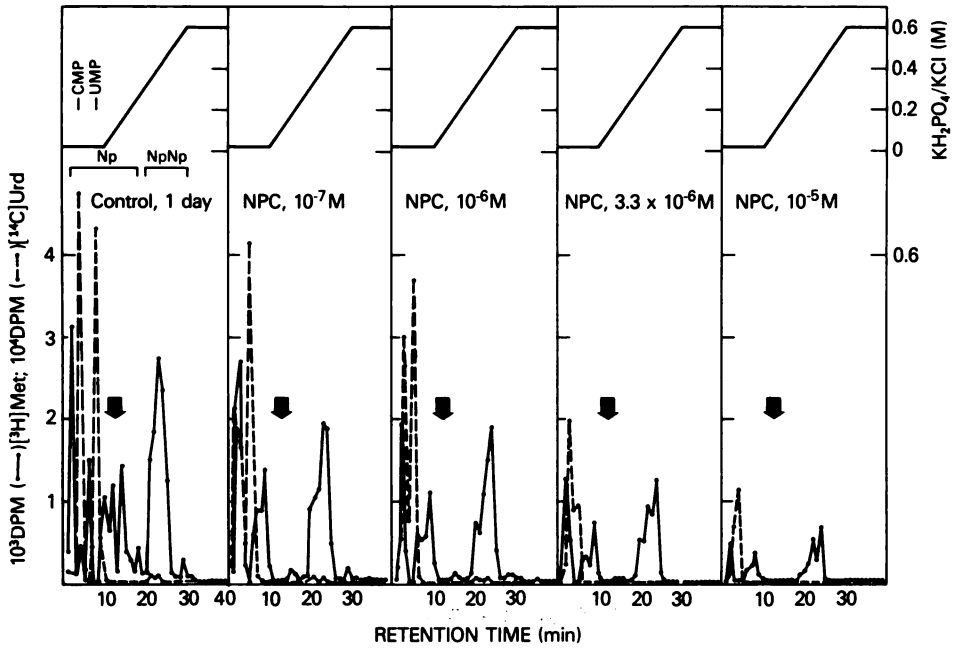

FIG. 5. *Anion exchange HPLC of RNase T_2 digests of RNA*

Cells were exposed for 24 hr to NPC and [^{3}H]methionine and [^{14}C]Urd and RNA was isolated and digested as described under Experimental Procedures. The arrow denotes base-methylated mononucleotides which are particularly susceptible to inhibition by NPC.

TABLE 2

RNase T_2 digestion of RNA

Cells were treated with NPC and [^{3}H]methionine and [^{14}C]Urd as described in Fig. 5. Each value is the mean of two experiments.

Drug treatment	Np fraction		NpNp fraction	
	^{3}H	^{14}C	^{3}H	^{14}C
	dpm			
Control	12,800	111,300	10,600	3,300
	% control			
NPC, 0.1 μM	75	80	70	73
NPC, 1 μM	45	69	56	57
NPC, 3.3 μM	29	44	38	39
NPC, 10 μM	14	16	16	18

days. The per cent reduction at 1 day approximated the decrease in cellular RNA synthesis (Fig. 3).

Metabolism of NPC. NPC was found previously to undergo anabolism to an AdoMet-like metabolite in colon carcinoma cells (4). Therefore, we examined the metabolism of NPC by reverse phase HPLC following alkaline phosphatase digestion of cell extracts to determine whether formation of this metabolite coincided with the effects of NPC on DNA and RNA methylation (Fig. 7). Incubation of cells for 24 hr with 3.3 μM NPC, the optimum concentration for inducing myeloid differentiation, resulted in the appearance of an NPC metabolite with the retention characteristics of AdoMet (NPC-Met). There was very little formation of the corresponding homocysteine metabolite (NPC-Hcy) and the remaining metabolites of NPC were nucleotide derivatives (appearing as the nucleoside following alkaline phosphatase digestion) as shown in Fig. 7. No free NPC was present in the cell extracts in the absence of alkaline phosphatase treatment. The time course for formation

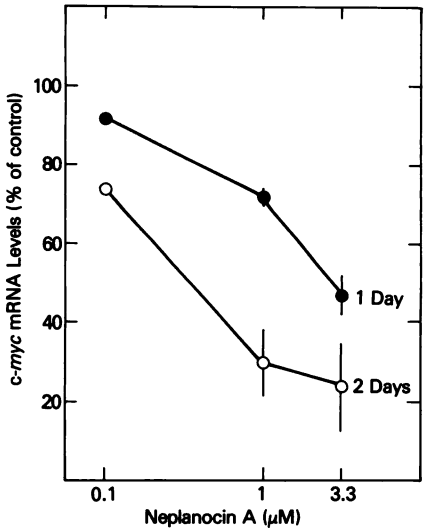

FIG. 6. *c-myc mRNA levels following treatment with NPC*

Cells were exposed for 24 or 48 hr to NPC and c-*myc* mRNA levels were determined by dot blot hybridization as described under Experimental Procedures. Each value is the mean ± standard error of three experiments.

of NPC-Met indicated that maximum formation occurred 1–2 days after continuous drug exposure with a rapid decline after 3 days (Fig. 8). Concurrent treatment of cells with NPC and the adenosine deaminase inhibitor dCF did not alter the metabolite profile of NPC (Table 3), and indicated that deamination was not limiting the anabolism of this drug.

DISCUSSION

NPC was found previously to inhibit RNA methylation and reduce cell viability in human colon carcinoma cell

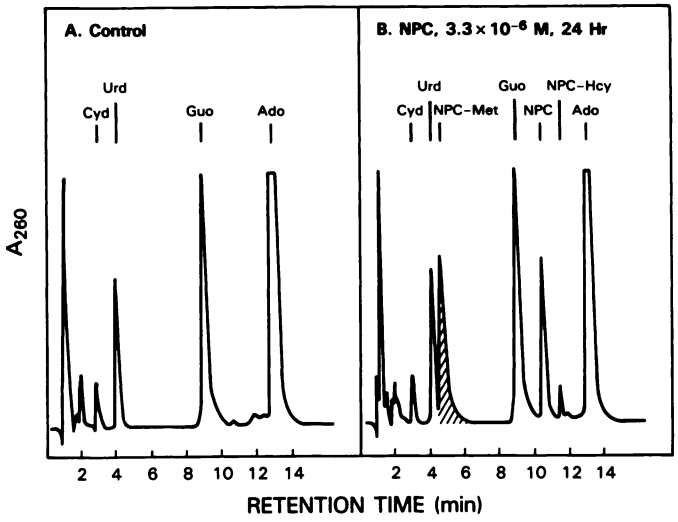

FIG. 7. *Reverse phase HPLC of metabolites of NPC*

Cells were exposed for 24 hr to 3.3 μM NPC. Trichloroacetic acid extracts were prepared, neutralized, and hydrolyzed with bacterial alkaline phosphatase. Metabolites were separated by reverse phase HPLC as described under Experimental Procedures. The shaded area represents NPC-Met.

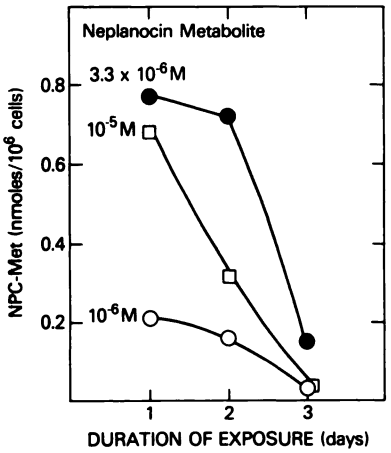

FIG. 8. *NPC-Met levels following time of exposure to NPC*

Cells were exposed continuously to varying concentrations of NPC and NPC-Met concentrations were determined by reverse phase HPLC as described under Experimental Procedures. Each value is the mean of two to three experiments.

line HT-29 via its conversion to a metabolite of AdoMet (4). The present study confirms the growth inhibitory activity of this drug and its ability to inhibit nucleic acid methylation in a human promyelocytic leukemia cell line, and further establishes that this carbocyclic adenosine analog is capable of inducing differentiation of this cell to a more mature myeloid phenotype. This effect was most dramatic for the appearance of myelocytic cells, and to a lesser degree neutrophil-like NBT-positive cells, indicating incomplete terminal differentiation. The appearance of the more differentiated cells was preceded by a modest reduction in DNA and RNA synthesis and by a greater suppression of RNA and DNA methylation. Coincident with these effects was a decrease in the expression of the amplified cellular oncogene, c-*myc*, which preceded the appearance of differentiated cells and the inhibition of cell growth. The reduction in c-*myc* mRNA has been noted previously in this cell line when induced to differentiate along the monocyte pathway with the vitamin D_3 metabolite, 1,25-dihydroxycholecalciferol (15) or along the myelocyte pathway by retinoic acid and DMSO (9). Whether the change in c-*myc* mRNA expression via its translation product or DNA and RNA methylation is a cause or effect of the differentiative response is not known; however, it seems likely that the effect of NPC on methylation results in reduced cell growth which in turn elicits differentiation. Indeed, there is evidence that many differentiating anticancer agents produce their effect by causing accumulation of cells largely in the G_1 phase of the cell cycle (16). We have in fact observed an even more rapid and pronounced response (90% NBT-positive cells) of HL-60 cells to myeloid differentiation[2] by the cyclopentenyl analog of cytidine, an inhibitor of CTP synthetase (17), and a drug which would be expected to block the G_1 to S phase transition.

NPC appears to be metabolized to an analog of AdoMet based on its retention time following HPLC. The chromatographic characteristics of this metabolite are identical to the synthetically prepared NPC-Met.[3] The identity of this metabolite was observed previously by Keller and Borchardt (18) in mouse L cells and by this laboratory in human colon carcinoma cell line HT-

[2] R. I. Glazer, K. C. Hartman, and M. C. Knode, manuscript in preparation.

[3] J. Linevsky and R. I. Glazer, unpublished results.

TABLE 3

Metabolism of NPC

Cells were treated with either 3.3 μM NPC alone or 3.3 μM NPC + 1 μM dCF for the indicated time interval. Each value is the mean ± standard error of three experiments.

Drug treatment	Exposure time	NPC-Met	NPC-Hcy	NPC nucleotides
	days		*nmol/10⁶ cells*	
NPC, 3.3 μM	1	0.85 ± 0.15	0.07 ± 0.02	0.21 ± 0.02
NPC, 3.3 μM + dCF	1	0.81 ± 0.12	0.06 ± 0.01	0.30 ± 0.03
NPC, 3.3 μM	2	0.61 ± 0.10	0.08 ± 0.01	0.11 ± 0.04
NPC, 3.3 μM + dCF	2	0.64 ± 0.11	0.07 ± 0.01	0.11 ± 0.03
NPC, 3.3 μM	3	0.11 ± 0.03	0.08 ± 0.03	0.008 ± 0.002
NPC, 3.3 μM + dCF	3	0.15 ± 0.04	0.03 ± 0.01	0.011 ± 0.003

29 (4). Since there was no accumulation of AdoHcy in either HT-29 (4) or HL-60 cells, NPC was not acting as an inhibitor of AdoHcy hydrolase as previously observed in vaccinia-infected L cells (5). Moreover, the relatively small levels of NPC-Hcy implies that NPC is not a substrate for AdoHcy hydrolase, but is rather a reflection of the poor methyl donor properties of NPC-Met. In addition to the NPC-Met metabolite, we and others have observed accumulation of nucleotides (predominantly the 5′-triphosphate) of NPC (4, 19). We have previously concluded that incorporation of NPC into RNA is a minor metabolic pathway for this drug, rendering it unlikely that it is involved in its antitumor activity. Since the 5′-triphosphate of NPC is also a substrate for AdoMet synthetase and is thus required for formation of NPC-Met, it is probable that the latter anabolite is the principal component involved in producing the cytotoxicity by NPC and the accompanying reduction in methylation. Whether NPC-Met simply acts competitively or as a tight-binding inhibitor of methyltransferases is not known. Further studies with synthetically prepared metabolites of NPC in cell-free systems will undoubtedly answer these questions.

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