MOLECULAR PHARMACOLOGY, 25:431-435

5-Methyl-2'-Deoxycytidine

Metabolism and Effects on Cell Lethality Studied with Human Leukemic Cells *in Vitro*

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Received August 10, 1983; Accepted December 6, 1983

SUMMARY

5-Methylcytosine (5MeCyt) is a possible regulator of eukaryotic gene transcription. We investigated whether this compound could be introduced into DNA from exogenous deoxyribonucleoside 5-methyl-2'-deoxycytidine (5MedCyd). High concentrations of 5MedCyd inhibited the growth of several types of human leukemic cell lines *in vitro*. However, the effect could be accounted for by dThd, a deamination product of 5MedCyd. We found that radioactivity from [*methyl*-^{14}C]5MedCyd and [2-^{14}C]5MedCyd was incorporated into DNA as thymidylate, and none was present as 5MeCyt. There are two conceivable metabolic pathways from 5MedCyd to thymidylate. The first consists of deoxycytidine or thymidine kinase and deoxycytidylate deaminase, and the second of sequential reactions catalyzed by deoxycytidine deaminase and thymidine kinase. No indication of the first pathway was demonstrable in human leukemic cells. We conclude that the DNA exclusion of 5MeCyt from exogenous 5MedCyd takes place because of powerful deoxycytidine deaminase activity in human malignant hematopoietic cells.

INTRODUCTION

5MeCyt[1] is the only modified base in mammalian DNA. DNA modification takes place during or shortly after semiconservative replication (1). There is no evidence that 5MeCyt would naturally be incorporated into DNA from the corresponding deoxyribonucleoside triphosphate. The only exception seems to be a bacteriophage, XP-12, in which all DNA cytosine is replaced by 5MeCyt (2, 3). On the other hand, we have observed in our preliminary studies that high concentrations of 5MedCyd *in vitro* were toxic to several kinds of human leukemic cells (4), although exogenous 5MedCyd seemed to be excluded from DNA (5).

The present investigation was undertaken to clarify the biochemical and pharmacological mechanisms responsible for the action of 5MedCyd at the cellular level. To that end, the influence of 5MedCyd on the growth of six human leukemia cell lines was investigated. Second, reversal of the 5MedCyd-induced growth inhibition in 3-day cultures of human leukemia cells using conventional pyrimidine deoxyribonucleosides was attempted. A third line of approach consisted of evaluation of the action of 5MedCyd on dThd-induced cytotoxicity. Moreover, we report here the results of experiments in which the cellular metabolism of two radioactive 5MedCyd derivatives, [2-^{14}C]5MedCyd and [*methyl*-^{14}C]5MedCyd, was investigated.

This work was supported by a grant from the Academy of Finland.

[1] The abbreviations used are: 5MeCyt, 5-methylcytosine; 5MedCyd, 5-methyl-2'-deoxycytidine; PBS, phosphate-buffered (pH 7.4) saline; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Cells. The human leukemic cell lines were a generous gift from Professor Leif Andersson, Department of Pathology, University of Helsinki. The main characteristics of the lines are as follows: BALL-1 is an acute lymphoblastic leukemia line with B cell differentiation (6). HL-60 is an acute promyelocytic leukemia line (7). JM is an acute T cell leukemia line. K-562 is a Philadelphia chromosome-positive cell line having erythroid and myeloid features (8). NALL-1 is an acute lymphoblastic leukemia cell line with neither B nor T cell differentiation (6). Raji represents a cell line derived from Burkitt's lymphoma (9).

The cells were maintained in 260-ml culture flasks (A/S Nunc, Roskilde, Denmark) in RPMI 1640 medium supplemented with 10% fetal calf serum (20% with HL-60), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). In maintaining cultures, new medium was added twice weekly. The cultures did not contain mycoplasma at the detection level reached by staining with Hoechst Compound 33258 (10).

Inhibition of leukemic growth in 3-day cultures. All assays were performed on Cooke microtiter V plates (Sterilin Ltd., Middlesex, England). The cultures were initiated with 2×10^4 cells in a volume of 200 μl/well. The given amounts of individual test substances were placed in the culture wells together with the cell suspension, and the cells were allowed to proliferate for 68–74 hr at 37° in humidified, CO_2-controlled (5%) atmosphere. At the end of the incubation, living cells

0026-895X/84/030431-05$02.00/0

were counted, using their ability to exclude trypan blue stain, in a hemocytometer (11).

Metabolic studies with radioactive 5MedCyd. We synthesized [2-^{14}C] 5MedCyd and [*methyl*-^{14}C]5MedCyd using a novel modification of Vorbrüggen's method starting from commercial dThd derivatives, as described in detail elsewhere (12). The incorporation of [*methyl*-^{14}C] dThd (specific activity 59.6 mCi/mmole), [2-^{14}C]5MedCyd (49 mCi/mmole), and [*methyl*-^{14}C]5MedCyd (55 mCi/mmole) was studied by exposing 1-ml subcultures of exponentially growing HL-60 cells to 0.1 μCi of the derivatives, individually, as follows: after 60 min of incubation at 37°, the cells were washed three times with PBS, the acid-soluble fraction was extracted with 0.2 N perchloric acid, RNA was hydrolyzed with 0.3 N KOH, DNA was hydrolyzed with 1 N perchloric acid at 80°, and the radioactivity incorporated into different fractions was counted as described in detail elsewhere (5).

It became evident that the "old" culture medium readily deaminated 5MedCyd. We tried to minimize this effect by performing the metabolic studies with cells freshly suspended in RPMI 1640 culture medium as follows: cells from master cultures were concentrated by centrifugation (300 × *g* for 10 min), the supernatant culture medium was carefully aspirated, and the loose cell pellet (8.4×10^6 BALL-1 cells or 6.6×10^6 HL-60 cells, from exponentially growing cultures) was resuspended in 100 μl of RPMI 1640 medium. The suspension was incubated with 0.1 μCi of [2-^{14}C]5MedCyd or with 0.1 μCi of [*methyl*-^{14}C]5MedCyd at 37° for 60 min, after which the cells were pelleted by centrifugation (1,000 × *g* for 10 min), the supernatant culture medium was isolated, and two volumes of ice-cold ethanol were added to it. This supernatant culture medium was kept at −20° for several hours, and the precipitate was removed by centrifugation (10,000 × *g* for 15 min). The clear ethanol-containing supernatant was used as the extracellular fraction. The cell pellet was extracted with 60% methanol at −20° for 16 hr. The methanol extract was centrifuged (10,000 × *g* for 15 min) and used as the intracellular soluble fraction. The methanol-extracted cell pellet was dried under a nitrogen flow, dissolved in 50 μl of proteinase K solution [proteinase K, 2 mg/ml; Tris-HCl (pH 7.5), 50 mM; NaCl, 150 mM; EDTA, 2 mM; sodium dodecyl sulfate, 0.5%], and incubated for 16 hr at 37°. The nucleic acids were separated using four successive phenol extractions, and the nucleic acids were precipitated with ethanol and washed three times in order to remove the phenol. RNA was hydrolyzed for 60 min at 37° with 0.3 N KOH. DNA was precipitated again with ethanol, and traces of RNA bases were removed by three washes with 70% ethanol. DNA was hydrolyzed with formic acid (30 min at 175°; see ref. 13). A similar hydrolysis was utilized for nucleosides and nucleotides. The bases were separated by using a novel one-plate TLC system making use of cellulose plates; the first ascending development was performed with butyl alcohol/ammonia/water (86/4/10) and the second ascending development with butyl alcohol/water (86/14). This method results in complete separation of Ade, Gua, Cyt, 5MeCyt, Ura, and Thy on a single plate, as will be described in detail elsewhere.[2] The other chromatographic resolutions of bases, nucleosides, and nucleotides involved the use of PEI-cellulose or cellulose plates as specified below.

Assay of extracellular deaminases. The capability of exponentially growing cell cultures to deaminate dCyd, 5MedCyd, and dCMP was investigated: to 100-μl subcultures, the radioactive substrates [5-^{3}H] dCyd (1 μCi/100 μl), [*methyl*-^{14}C]5MedCyd (0.1 μCi/100 μl), and [U-^{14}C]dCMP (0.1 μCi/100 μl) were individually added. The chemical concentration of each substrate was adjusted to 20 μM. The substrates were incubated at 37° for 0, 10, or 30 min, after which the cells were pelleted by centrifugation (10,000 × *g* for 2 min), the supernatant was combined with 200 μl of ice-cold ethanol, and the ethanol precipitate was removed by centrifugation (10,000 × *g* for 15 min). The reaction products were separated from the respective substrates by TLC: dCyd/dUrd and 5MedCyd/dThd on cellulose plates (butyl alcohol/water = 86/14 development system), and dCMP/dUMP on PEI-plates with a formic acid development system (14).

Chemicals. 5MedCyd, 5MeCyt, 5MedCMP, proteinase K, and highly polymerized calf thymus DNA were purchased from Sigma Chemical Company (St. Louis, Mo.). Radioactive nucleosides were from The Radiochemical Centre (Amersham, United Kingdom), and other bases, nucleosides, and nucleotides were from Calbiochem-Behring Corporation (La Jolla, Calif.). Other sources of chemicals were as follows: glutamine, penicillin, streptomycin, fetal calf serum, and RPMI 1640 culture medium, Gibco Europe Ltd. (Middlesex, England); bovine serum albumin, Armour Pharmaceutical Company Ltd. (Eastbourne, England); TLC plates, Merck (Darmstadt, Federal Republic of Germany).

[2] J. A. Vilpo. Simple one-plate TLC separation of DNA bases including 5-methylcytosine, manuscript in preparation.

RESULTS

Inhibition of cell growth in 3-day cultures. 5MedCyd had a relatively weak inhibitory effect on the growth of different types of leukemic cell lines, as shown in Table 1. Nevertheless, inhibition was demonstrated in all cell lines, and the highest 5MedCyd concentration (2.5 mM) reduced the cell harvest by 50% or more in HL-60, JM, K-562, NALL-1, and Raji cell cultures. The inhibition in BALL-1 culture was weaker (Table 1).

Reversal of 5MedCyd-induced growth inhibition by conventional pyrimidine deoxyribonucleosides. The mode of action of 5MedCyd in 3-day cultures was further characterized with HL-60 cells, which appeared to be sensitive to 10–100 μM 5MedCyd (Table 1). dCyd slightly increased the cell numbers when added to cultures containing 5MedCyd. This effect appeared to be dependent on the concentration of dCyd (Fig. 1). No similar effect was observed with dThd.

Comparison of dCyd and 5MedCyd as antagonists of dThd-induced growth inhibition of HL-60 cells. dThd is known to inhibit the proliferation of HL-60 cells in culture (4, 15). As a rule, dThd-induced toxicity is at least partially rescuable with dCyd (15). We tested here whether dCyd rescue could be demonstrated under our culture conditions and whether 5MedCyd could substitute for dCyd in the reversal of toxicity of dThd. 100 μM and 1 mM dThd inhibited the growth of HL-60 cells in 3-day cultures, as shown in Table 2. Partial rescue was recorded with dCyd. Improvement in cell yields, caused by dCyd, was best demonstrable (i.e., 58%) when the

TABLE 1

Inhibition of growth of six different hematopoietic cell lines by 5MedCyd

The cells were grown in microplate cultures in the presence of 5MedCyd. After 3 days the number of living cells was counted hemocytometrically, using trypan blue exclusion. Values are means of four cultures ± standard deviation.

Cell line	Living cells		
	5MedCyd, 25 μM	5MedCyd, 250 μM	5MedCyd, 2.5 mM
	% of nontreated controls		
BALL-1	86 ± 23	60 ± 17	60 ± 8
HL-60	70 ± 5	28 ± 5	24 ± 6
JM	106 ± 8	81 ± 9	17 ± 8
K-562	85 ± 6	92 ± 10	50 ± 4
NALL-1	101 ± 11	98 ± 5	39 ± 1
RAJI	94 ± 14	66 ± 8	10 ± 2

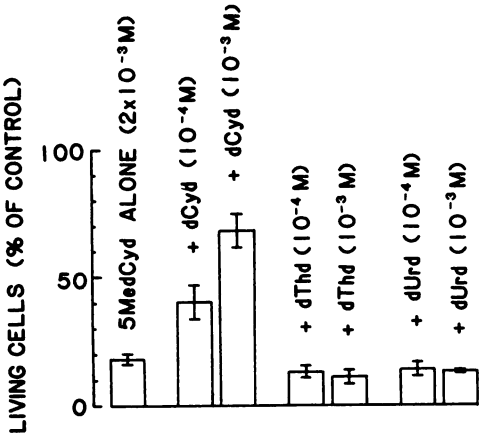

FIG. 1. *Effect of dCyd, dThd, and dUrd on growth of 5MedCyd-treated cultures*

HL-60 cells were allowed to proliferate for 3 days in microplate cultures in the presence of 5MedCyd (2 mM) alone and together with two different concentrations of dCyd, dThd, and dUrd. Mean results of three cultures (± standard deviation) are presented.

inhibitory concentration of dThd was 1 mM and the dCyd concentration was also 1 mM (Table 2). On the other hand, 5MedCyd did not have such an effect; the latter compound possibly potentiated the growth-inhibitory action of dThd (Table 2).

Deamination of 5MedCyd in culture medium. No deaminating activity was observed in our fresh complete culture medium (see Materials and Methods), nor was it present in the fetal calf serum, which could be the biological source of a deaminase. On the other hand, in the culture medium of well-proliferating cells, a 5MedCyd deaminase was readily demonstrable. When HL-60 cell cultures were exposed to 5MedCyd (1 mM), 2% of the compound was deaminated to dThd over 2 hr and 51% over 72 hr of exposure. dCyd and 5MedCyd, but not dCMP, appeared to be good substrates for that deaminase, as demonstrated with HL-60 cell cultures (Fig. 2).

Metabolism of [methyl-^{14}C]5MedCyd and [2-^{14}C]5MedCyd in BALL-1 and HL-60 cells. The radioactivity

TABLE 2

Modulation of dThd-induced cytotoxicity by dCyd versus 5MedCyd

HL-60 cells were grown for 3 days in microplate cultures in the presence of dThd alone and with different concentrations of dCyd or 5MedCyd, after which the living cells were counted hemocytometrically. Values are means of three cultures ± standard deviation.

Additional treatment	Living cells		
	No dThd added	dThd, 100 μM	dThd, 1 mM
	% of nontreated controls		
None		47 ± 4	26 ± 2
dCyd, 10 μM	—	64 ± 2	27 ± 1
5MedCyd, 10 μM	87 ± 3	54 ± 5	27 ± 4
dCyd, 100 μM	—	63 ± 3	23 ± 2
5MedCyd, 100 μM	27 ± 4	24 ± 1	24 ± 1
dCyd, 1 mM	—	57 ± 3	41 ± 4
5MedCyd, 1 mM	23 ± 3	17 ± 1	17 ± 4

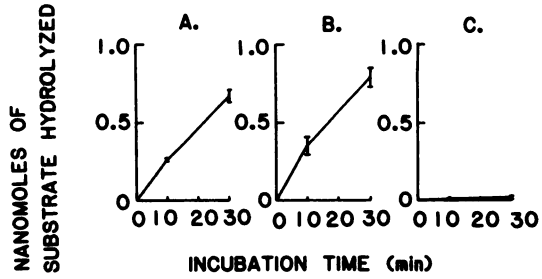

FIG. 2. *Hydrolysis of 5MedCyd, dCyd, and dCMP by deaminases present in the culture medium of HL-60 cells*

Aliquots (100 μl) of exponentially growing HL-60 cell cultures were exposed for 0, 10, and 30 min to 20 μM [*methyl*-^{14}C]5MedCyd (A), 20 μM [5-^{3}H]dCyd (B), and 20 μM (U-^{14}C)dCMP (C). The extent of deamination was calculated on the basis of chromatographic resolution of respective substrates and end-products: A, 5MedCyd versus dThd; B, dCyd versus dUrd; C, dCMP versus dUMP.

from [*methyl*-^{14}C]5MedCyd and [2-^{14}C]5MedCyd was abundantly incorporated in BALL-1 and HL-60 cells. A quantitative comparison of incorporation of dThd and 5MedCyd is illustrated in Table 3. The rate of incorporation of [*methyl*-^{14}C]5MedCyd into HL-60 cells was identical with that of [2-^{14}C]5MedCyd. The rate of incorporation of [*methyl*-^{14}C]dThd into DNA did not appear to be faster than that of 5MedCyd analogues. However, the radioactive concentration of dThd-derived activity in the acid-soluble pool was a little lower than the 5MedCyd-derived one. We also isolated RNA and protein fractions, but very little, if any, radioactivity was present in them.

The intracellular metabolism of 5MedCyd was further investigated by minimizing the effect of extracellular deaminases; fresh culture medium was substituted for the old deaminase-containing medium just before the cells were exposed to radioactive 5MedCyd derivatives. Nevertheless, extracellular deamination of 5MedCyd was observed, and after the 60-min incubation period only 20–30% of the radioactivity in BALL-1 cell culture medium and 3–5% of the radioactivity in HL-60 cell culture medium was in the form of 5MedCyd; the rest was dThd. No radioactive 5MeCyt was present in the DNA of [^{14}C]5MedCyd-exposed cells, although radioactive Thy was readily demonstrable and no differences in this respect

TABLE 3

Incorporation of [methyl-^{14}C]5MedCyd, [2-^{14}C]5MedCyd, and [methyl-^{14}C]dThd into HL-60 cells

Exponentially growing HL-60 cell cultures were exposed for 60 min to 0.1 μCi/ml of [*methyl*-^{14}C]dThd, [*methyl*-^{14}C]5MedCyd, or [2-^{14}C]5MedCyd having approximately the same specific activity (see Materials and Methods). The acid-soluble fraction and the DNA fraction were isolated and radioactivity was counted. Values are means (± standard deviation) of three determinations.

Fraction	Incorporation		
	[*methyl*-^{14}C] dThd	[*methyl*-^{14}C] 5MedCyd	[2-^{14}C] 5MedCyd
	pmoles/10^6 cells		
Acid-soluble	1.5 ± 0.1	4.4 ± 0.3	4.5 ± 0.6
DNA	39.0 ± 1.9	37.0 ± 1.6	42.0 ± 4.0

were noted between the *methyl-* or 2-labeled 5MedCyd isotopes (Table 4).

In order to clarify the metabolism of 5MedCyd, we chose BALL-1 cells for further studies, because BALL-1 culture medium appeared to deaminate 5MedCyd to a lesser extent than HL-60 cell culture medium. Radiochromatography of the methanol extract of 5MedCyd-exposed BALL-1 cells demonstrated the absence of 5MedCMP (Fig. 3). Base analysis of this extract performed after complete formic acid hydrolysis revealed that only 10% of the radioactivity was in 5MeCyt-containing substrates. The main base was Thy, comprising 90% of [2-^{14}C]5MedCyd-derived radioactivity. This means that the great majority of the radioactivity in the compounds illustrated in Fig. 3 must have been in the form of Thy. Moreover, it is highly likely that the radioactivity which co-chromatographed with dTTP and dTDP was in the form of Thy, since no 5MeCyt was present in the DNA of these cells (cf. Table 4).

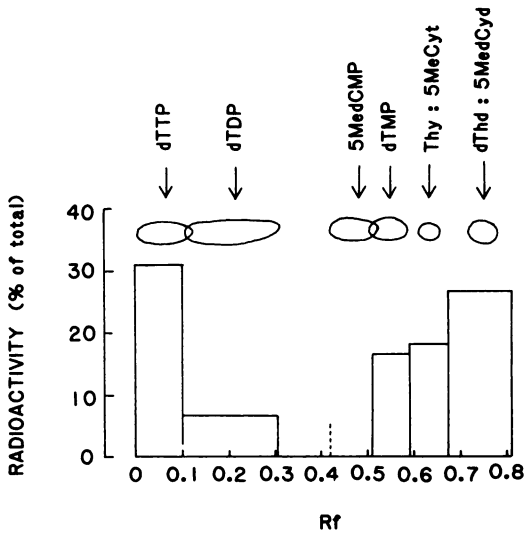

FIG. 3. *Radiochromatography of methanol extract obtained from BALL-1 cells after a 60-min exposure to [2-^{14}C]5MedCyd*

Exponentially growing BALL-1 cells (8.4×10^6) were placed in 100 μl of fresh culture medium containing 0.1 μCi of [2-^{14}C]5MedCyd and incubated for 60 min at 37°. The cells were washed once with PBS and extracted overnight at −20° with 60% methanol. A sample of methanol extract was chromatographed on PEI-cellulose plates with dTTP, dTDP, dTMP, 5MedCMP, dThd, 5MedCyd, Thy, and 5MeCyt as internal markers. The chromatograms were developed with 0.4 N LiCl. The positions of the markers are illustrated.

DISCUSSION

Biological methylation of eukaryotic DNA appears to be an important mechanism for regulation of the transcriptional activity of genes (16). The present investigation was undertaken to clarify the possibility of methylating DNA by introducing DNA-5MeCyt residues from corresponding deoxyribonucleoside. It was known in advance that, although 5MedCTP may be a good substrate for DNA polymerase (17), natural DNA modification takes place after incorporation of dCMP into DNA via semiconservative replication (1).

We demonstrated that 5MedCyd is toxic in high concentrations to several types of human leukemic cell lines. Our results are in accordance with those obtained by Adams and co-workers (18): they did not observe appreciable inhibition with 10 μM 5MedCyd when tested against Chinese hamster ovary cells. No higher concentrations were tested by them. The toxicity of 5MedCyd was evidently based on the deamination of 5MedCyd to dThd.

TABLE 4

Incorporation of [^{14}C]5MedCyd into DNA-bases of human leukemic cells

Exponentially growing BALL-1 cells (8.4×10^6) and HL-60 cells (6.6×10^6) were suspended in 100 μl of fresh culture medium containing 0.1 μCi of [*methyl*-^{14}C]5MedCyd or 0.1 μCi of [2-^{14}C]5MedCyd. The cells were incubated for 60 min, washed once with PBS, and extracted overnight with 60% methanol, after which the cells were digested for 16 hr with proteinase K, extracted with phenol, hydrolyzed for 14 hr with 0.3 N KOH, and washed four times with ethanol. DNA was hydrolyzed to its constituent bases with formic acid. The bases were resolved chromatographically on cellulose plates with internal markers as described under Materials and Methods. The marker spots were cut out and the radioactivity was counted. The background activity was 20 ± 2 cpm (± SD).

Base	Incorporation		
	HL-60		BALL-1
	[*methyl*-^{14}C] 5MedCyd	[2-^{14}C] 5MedCyd	[2-^{14}C] 5MedCyd
	cpm/6.6 × 10^6 cells		*cpm/8.4 × 10^6 cells*
Gua	20	22	20
Cyt	20	22	18
5MeCyt	20	16	19
Ade	24	19	22
Ura	20	18	21
Thy	4968	5416	6167

Our results revealed that the base 5MeCyt is not incorporated into DNA as 5MeCyt but that the compound is deaminated to Thy before its incorporation into DNA. We could have demonstrated if approximately 0.1% of [^{14}C]5MedCyd-derived radioactivity had become incorporated into DNA as 5MeCyt, but this was not the case; only radioactive Thy was recovered. This finding confirms our previous results and extends the sensitivity by which we could have detected 5MedCyd-derived 5MeCyt in DNA by a factor of 10 (5). Although exogenous 5MedCyd is effectively excluded from DNA in human leukemic cells, there still is a possibility of rapid excision of 5MeCyt from a "wrong" position in DNA. DNA demethylating activity has been demonstrated in hematopoietic cells (19). However, it cannot be responsible for the exclusion of exogenous 5MedCyd from DNA. If that were the case, some of the radioactivity derived from [2-^{14}C]5MedCyd should have been demonstrable in the DNA-cytosine of HL-60 cells.

DNA-Thy was the main metabolic end-product of 5MedCyd. There are two conceivable metabolic pathways from 5MedCyd to DNA-Thy: (a) sequential reactions catalyzed by deoxycytidine kinase and deoxycytidylate deaminase to thymidylate, and (b) sequential reactions catalyzed by deoxycytidine deaminase, and thymidine kinase to thymidylate. There was no indication of the former pathway. Namely, no radioactive 5MedCMP was found in the cells exposed to [^{14}C]5MedCyd derivatives.

Another observation, favoring the prominence of the latter pathway, was the abundance of [^{14}C]5MedCyd-derived [^{14}C]dThd in the exposed cells. Furthermore, we demonstrated an extracellular enzymatic activity capable of deaminating 5MedCyd with reaction kinetics similar to that of the enzyme deaminating dCyd; this cell-derived enzyme did not use dCMP as its substrate at all.

ACKNOWLEDGMENT

We thank Mrs. Leena Vilpo for technical assistance.

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