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EXCLUSION OF EXOGENOUS 5-METHYL-2′-DEOXYCYTIDINE FROM DNA IN HUMAN LEUKEMIC CELLS

A STUDY WITH [2-^{14}C]- AND [METHYL-^{14}C]5-METHYL-2′-DEOXYCYTIDINE

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Abstract—Modification of DNA-cytosine by a 5-methyl group is thought to be an important mechanism which regulates the expression of eukaryotic genes. This modification takes place after semiconservative replication. There is very little evidence, if any, that 5MeCyt could be naturally incorporated into mammalian DNA in semiconservative replication. We have clarified the possibility of incorporating 5MedCyd pharmacologically into human leukemic cells *in vitro*. To this end, we developed a novel small-scale synthesis method for ^{14}C-labeled 5MedCyd starting from commercially available [^{14}C]dThd derivatives. Particular attention was focused upon possible incorporation of radioactive 5MedCyd derivatives into the acid-soluble cellular fraction as well as into nucleic acids and protein in human cells. The results showed that [2-^{14}C]- and [methyl-^{14}C]5MedCyd were incorporated into human leukemic cells to a similar extent. The radioactivity originating from these compounds was incorporated mainly into the acid-soluble pool and nucleic acids. The exact nature of the intracellular radioactive molecules in RNA is not known, but the radioactive label in DNA hydrolyzate co-chromatographed exclusively with thymine. Hence, 5MedCyd is deaminated to thymidine before incorporating into DNA. This deamination had taken place already (partially) in the culture medium. Human leukemic cells do effectively protect their DNA from incorporation of exogenous 5MedCyd.

Post-synthetic modification of almost all classes of biological macromolecules is a well-known phenomenon. This includes methylation of nucleic acids. In general, there are only two naturally occurring methylated (minor) bases in DNA: N^6-methyladenine and 5MeCyt† [1, 2]. 5MeCyt is the only minor methylated fraction of the total bases in eukaryotes and higher plants, varying from as low as 0.17 mole % MeCyt (of the cytosines) in insects up to 8 mole % MeCyt in mammals. In certain higher plants the level may rise to as high as 50% (for a review, see Ref. 3). 5MeCyt is also a well-known component of RNA, e.g. it is found in high concns in rat and rabbit liver transfer RNA [4, 5].

During the replication of mammalian genomes more than 10^7 cytosines of the newly synthesized strand become enzymatically methylated by *S*-adenosylmethionine : DNA(cytosine-5) - methyltransferases, presumably via a semiconservative copying mechanism [6]. The biological significance of DNA methylation in eukaryotic cells is not completely understood. There is, however, evidence suggesting that DNA methylation could be a specific means for the regulation of gene transcription [3]. In this view, most of the data currently available link the methylation of a gene to a decrease in its transcriptional expression [7–10].

5MedCyd triphosphate is a good substrate for *E. coli* DNA polymerase I, when tested in a cell-free system *in vitro* [11]. Thus, it is conceivable that 5MedCyd could be incorporated into DNA in semiconservative replication. The prerequisite is that the compound enters the cell and that it is phosphorylated to a corresponding deoxyribonucleoside triphosphate.

The present study was undertaken to examine the possibility of incorporation of 5MedCyd into human leukemic cells. To this end, a novel modification of the procedure described by Vorbrüggen *et al.* [12] was developed for the synthesis of [2-^{14}C]- and [methyl-^{14}C]5MedCyd. We show here that the radioactivity originating from ^{14}C-labeled 5MedCyd derivatives is readily incorporated into the nucleic acids of two human leukemic cell lines, when assayed in short-term cultures *in vitro*.

MATERIALS AND METHODS

Chemicals. RPMI 1640 culture medium, fetal calf serum, glutamine and penicillin-streptomycin were from Gibco Europe Ltd (Middlesex, U.K.); 5MedCyd, highly polymerized calf thymus DNA, proteinase K and bovine serum albumin from Sigma Biochemical Co. (St. Louis, MO); thymidine and

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† Abbreviations: 5MedCyd, 5-methyl-2′-deoxycytidine; 5MeCyt, 5-methylcytosine.

bases from Calbiochem-Behring Corp. (La Jolla, CA); [methyl-^{14}C]thymidine, [2-^{14}C]thymidine, [methyl-^{3}H]thymidine, L-[U-^{14}C]leucine and [5-^{3}H]uridine from the Radiochemical Centre (Amersham, U.K.); cellulose TLC plates and formamide from Merck (Darmstadt, F.R.G.); and hexamethyldisilazane from Fluka A.G. (Buchs, Switzerland).

Preparation of ^{14}C-labeled 5MedCyd derivatives. [2-^{14}C]- and [methyl-^{14}C]5MedCyd were prepared from formamide plus [2-^{14}C]- and [methyl-^{14}C]dThd, respectively, essentially according to the procedure of Vorbrüggen *et al.* [12]. In short, [^{14}C]dThd, hexamethyldisilazane and formamide were heated in a glass ampoule at 140° for 76 h and the reaction mixture was refluxed with absolute methanol for 3 h. The reaction products were rigorously purified from the mother compounds and from the side products on TLC plates. The method will be described in detail elsewhere.*

Cell lines. We used human lymphoblastic cell lines JM and NALL-1. JM represents an acute lymphoblastic leukemia with T-cell differentiation [13], and NALL-1 is derived from an acute lymphoblastic leukemia with neither B- nor T-cell differentiation [14]. The cells were maintained in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Other conditions were a CO_2-controlled (5%) humidified atmosphere at 37°. The cultures did not contain *Mycoplasm* at the detection level reached by staining with Hoechst Compound 33258 [15].

Incorporation studies. The incorporation of the various radioactive precursors was studied in 1 ml short-term subcultures containing 0.4×10^6–1.4×10^6 cells as follows: 1-ml aliquots from a master culture were placed in plastic culture tubes containing an appropriate amount of the radioactive precursor at the following final concns: [2-^{14}C]5MedCyd (49 mCi/mmole, 0.2 μCi/ml), [methyl-^{14}C]5MedCyd (55 mCi/mmole, 0.2 μCi/ml), [methyl-^{3}H]thymidine (47 Ci/mmole, 0.2 μCi/ml), [5-^{3}H]uridine (28 Ci/mmole, 2.0 μCi/ml), and L-[U-^{14}C]leucine (720 mCi/mole, 0.2 μCi/ml). After 60 min incubation at 37° the cells were washed 3 times with ice-cold phosphate-buffered (pH 7.4) saline. The cells were then dissolved in 666 μl of water containing 50 μg of highly polymerized carrier DNA and 250 μg of carrier bovine serum albumin. The tubes were incubated in an ice-bath and 333 μl of 0.6 N perchloric acid were added. Nucleic acids and proteins were precipitated for 15 min and the supernatant was taken as an acid-soluble fraction after centrifugation (1000 *g*, 10 min). The precipitates were washed twice with 3 ml of 0.2 N perchloric acid. The precipitates were then dissolved in 0.5 ml of 0.3 N KOH and RNA was hydrolyzed for 60 min at 37° [16]. The hydrolysate was acidified by adding perchloric acid to a final concn of 0.2 N, and DNA and protein were precipitated. After centrifugation (1000 *g*, 10 min) the supernatant was taken to a counting vial and the precipitate was washed once with 0.5 ml of 0.2 N perchloric acid. The washings were combined with the RNA hydrolysates. DNA was extracted twice (80°, 20 min) with 1 N perchloric acid, and taken to a separate counting vial, after which the residual protein was dissolved in 20 μl of 1 N NaOH and 1 ml of water. The radioactivity in the various cellular compartments was counted in a scintillation spectrophotometer using internal standardization with ^{3}H and ^{14}C standards.

Stability of radioactive 5MedCyd in culture medium. [2-^{14}C]- and [methyl-^{14}C]5MedCyd were added to 1-ml cultures of NALL-1 cells (0.1 μCi/ml) and the cells were pelleted (10,000 *g*, 2 min at +2°) after 0, 5, 10, 30 and 60 min of incubation at 37°. The supernatant was placed in an ice-bath and 2 vols of ethanol were added. After 15 min, a centrifugation (10,000 *g*, 15 min) was performed and samples of the supernatant were chromatographed with deoxyribonucleoside markers on cellulose plates with two systems: butanol–water (86:14) and butanol–methanol–water–ammonia (60:20:20:1), after which the individual marker spots were cut off and the radioactivity counted.

Purification, hydrolysis and base analysis of cellular DNA. The NALL-1 cells (1.6×10^7 in 1 ml of culture medium) were incubated for 60 min at +37° in the presence of 2.5 μCi of [2-^{14}C]- or [methyl-^{14}C]5MedCyd. The cells were then washed twice with ice-cold PBS and the cell pellets were 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; SDS, 0.5%) and incubated for 16 hr at +37°. The nucleic acids were separated by four successive phenol extractions. Then the nucleic acids were precipitated with ethanol and washed three times in order to remove phenol. RNA was hydrolyzed for 16 hr 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° (Refs. 7 and 8)] and bases were separated chromatographically on cellulose plates.

RESULTS

Stability of radioactive 5MedCyd in culture medium

[2-^{14}C]- and [methyl-^{14}C]5MedCyd were gradually hydrolyzed to corresponding dThd derivatives in the culture medium of NALL-1 cells (Table 1). This hydrolysis was completed during the 60-min incubation period. Nevertheless, some 5MedCyd was available for the cells during the first 30 min as indicated, and this nucleoside was also incorporated into the acid-soluble pool of the cells (results not shown).

Incorporation of radioactive precursor molecules

The incorporation of [2-^{14}C]- and [methyl-^{14}C]5MedCyd into various intracellular compartments is illustrated in Fig. 1. Incorporation into the acid-soluble fraction and DNA is evident in both JM and NALL-1 cell lines. Furthermore, a considerable amount of radioactivity was also present in the RNA fraction of NALL-1 cells. If a high molar excess of non-radioactive 5MedCyd (10^{-3} M) was added to the incubation mixture, the intracellular radioactivity decreased more than 99% in all examined compart-

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Table 1. Distribution of radioactivity between 5MedCyd and dThd in the culture medium in [^{14}C]5MedCyd-treated cultures

Incubation time (min)	Radioactivity (% of total)			
	[2-^{14}C]5MedCyd		[Methyl-^{14}C]5MedCyd	
	5MedCyd	dThd	5MedCyd	dThd
0	100	0	100	0
5	51	49	50	50
10	34	66	30	70
30	8	92	4	96
60	0	100	0	100

ments in JM cells. Figure 1 also illustrates the incorporation of some well-known precursor molecules. It can be seen that most of the radioactivity derived from [methyl-^{3}H]dThd was present in DNA, most of that from [5-^{3}H]Urd entered RNA, and most radioactivity from L-[U-^{14}C]leucine entered the protein fraction although some spill-over can be seen in the DNA-containing hydrolysate.

Base analysis of DNA

The DNA base analysis detected that the label derived from either [2-^{14}C]- or [methyl-^{14}C]5MedCyd was exclusively in thymine (Table 2). The detection limit of our procedure was less than 1%. In other words, we could have detected it if 1% of the label in DNA had been MeCyt.

DISCUSSION

Modification of DNA–cytosine by a 5-methyl group could very probably be involved in the regulation mechanism of the expression of eukaryotic genes, although this concept is still poorly understood [3]. The modification of DNA takes place after semiconservative replication and there is very little evidence, if any, that 5MedCyd would be naturally incorporated into eukaryotic DNA from a corresponding deoxyribonucleoside triphosphate. The only exception so far discovered in nature seems to be a bacteriophage, XP-12, in which all the DNA–cytosine is replaced by 5MeCyt [19, 20]. We have done some pilot experiments in order to test the possible cytotoxicity of non-radioactive 5MedCyd towards various human leukemic cell lines *in vitro*.

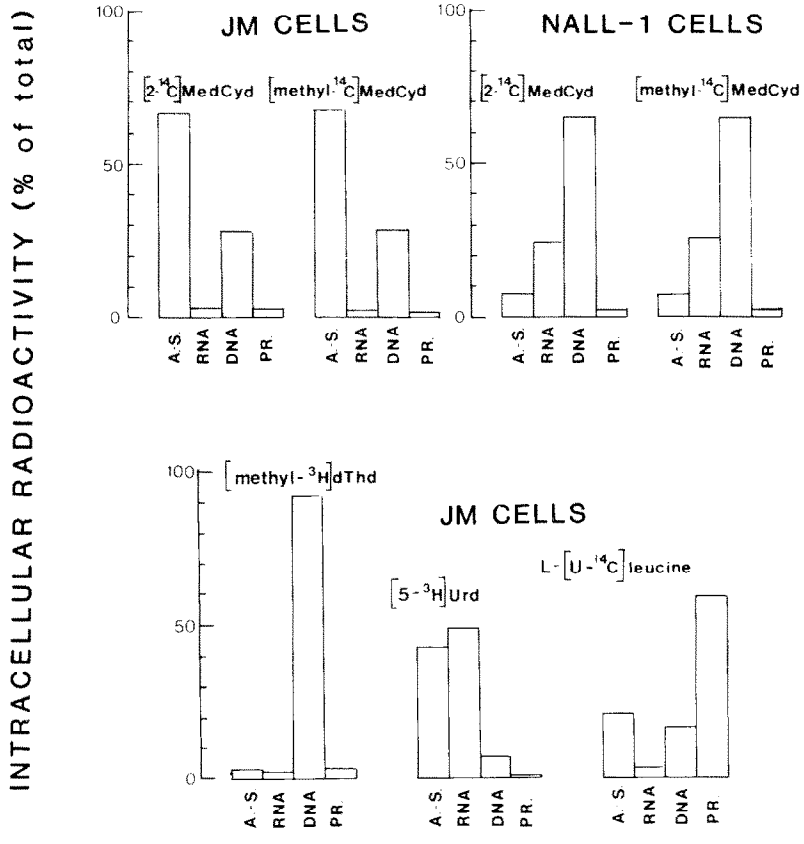

Fig. 1. Distribution of radioactivity derived from given precursor molecules in different intracellular compartments at the end of a 60-min incubation period *in vitro*. Above: incorporation of [2-^{14}C]- and [methyl-^{14}C]5MedCyd radioactivities into JM (left) and NALL-1 cells (right). Below: incorporation of the reference compounds [methyl-^{3}H]dThd, [5-^{3}H]Urd, and L-[U-^{14}C]leucine into JM cells. Each column represents the average of three assays. A.-S. means acid-soluble fraction and PR. means protein.

Table 2. Distribution of radioactivity between different DNA bases in [^{14}C]5MedCyd-treated NALL-1 cells

Base	Radioactivity (cpm/3.4 × 10^6 cells)	
	[2-^{14}C]5MedCyd	[Methyl-^{14}C]5MedCyd
Adenine	20	20
Guanine	25	22
Cytosine	22	19
Uracil	15	22
Thymine	1197	1042
5MeCyt	20	24

The cytotoxicity has been readily demonstrable with high concns of 5MedCyd.*

The present study was undertaken to clarify the possibility of incorporating 5MedCyd into DNA, and thus possibly affecting the putative mechanism which might be operative in the regulation of gene transcription in human cells. To this end, we synthesized two radioactive derivatives of 5MedCyd. The versatility of the radioactive 5MedCyd compounds was seen in the incorporation studies. Quantitatively, the incorporation of both 5MedCyd derivatives into different subcellular compartments was similar. Furthermore, the radioactivity was incorporated mainly into the acid-soluble fraction as well as into nucleic acids. This pattern was different from that seen with [^{3}H]dThd. Hence, it is unlikely that all the 5MedCyd was deaminated to dThd before incorpation into cells. The relative size of the radioactive acid-soluble pool in JM cells, derived from [^{14}C]5MedCyd, was much higher, and RNA radioactivity much lower than in NALL-1 cells. The nucleic acid metabolism of these cell lines is not known in detail and we do not know whether the difference was based on some differences in metabolism *per se*, or whether the difference reflected different growth activities at the time of sampling.

Our results indicate that, although 5MedCyd has potent pharmacological effects on human leukemic cells *in vitro*, the compound itself is not incorporated into cellular DNA to a significant extent. Hence, the toxicity of the compound is explicable in terms of molecular modification. In fact, we demonstrated a potent (ecto)deaminase in the culture medium, which completely hydrolyzed 5MedCyd to dThd in 30–60 min.

* A. Jekunen and J. Vilpo, unpublished results.

The strict exclusion of 5MedCyd from DNA is an important mechanism, which maintains the integrity of genetic information and prevents a possible interference of pharmacological DNA methylation by 5MedCyd with the physiological (post-replicative) DNA methylation by DNA methylases.

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