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5-Hydroxymethyl-2′-deoxyuridine. Cytotoxicity and DNA Incorporation Studied by Using a Novel [2-^{14}C]-Derivative with Normal and Leukemic Human Hematopoietic Cells

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5-Hydroxymethyl-2′-deoxyuridine is a biologically active thymidine analogue. This investigation was aimed at characterizing the cytotoxicity of 5-hydroxymethyl-2′-deoxyuridine and its incorporation into DNA. Fifty percent inhibition of cellular proliferation, assessed by incorporation of [U-^{14}C]-L-leucine in vitro, was caused by 1.7–5.8×10^{-5} M 5-hydroxymethyl-2′-deoxyuridine in seven human leukemia cell lines. Higher concentrations of 5-hydroxymethyl-2′-deoxyuridine, *i.e.* 6–8×10^{-5} M, were required for a comparable inhibition in human PHA-stimulated peripheral blood lymphocytes. A new synthesis procedure for [2-^{14}C]5-hydroxymethyl-2′-deoxyuridine was developed. The net incorporation of [2-^{14}C]5-hydroxymethyl-2′-deoxyuridine into DNA of hematopoietic cells was low. The possibility of a repair mechanism for 5-hydroxymethyluracil bound to DNA is discussed.

The cytostatic properties of deoxyuridines substituted at the carbon-5 position are well known. The compound 5-hydroxymethyl-2′-deoxyuridine (5HmdUrd) is a biologically active thymidine analogue which reportedly inhibits growth and cell division in *Escherichia coli*[1] and various mammalian cells in culture; the minimal inhibitory concentrations for different cell lines, such as Vero, BHK 21/C13, HEF, and L1210 are in the order of 10^{-6} to 10^{-5} M.[2,3]

The mode of action of 5HmdUrd on cellular metabolism is not known in detail. 5-Hydroxymethyluracil is a natural constituent of DNA in certain *Bacillus subtilis* phages, substituting for thymine.[4–6] Some 5-hydroxymethyluracil is also found in the DNA of certain *Dinoflagellates*.[7] Hence, the compound can be incorporated into DNA and its presence *per se* does not affect the various functions of DNA. The current interest in 5HmdUrd is strengthened by two recent reports; 5-hydroxymethyluracil could be detected in mammalian cell DNA after ionizing radiation and as a result of tritium transmutation,[8] and 5-hydroxymethyluracil may be excluded from DNA in a similar fashion as uracil.[9]

This investigation was undertaken to clarify the influence of 5HmdUrd on the proliferation of seven human leukemic cell lines and on peripheral blood PHA-stimulated lymphocytes in 3-day cultures *in vitro*. Furthermore, we prepared [2-^{14}C]-5HmdUrd using a new synthesis procedure described in this paper. We report on the incorporation of 5HmdUrd into DNA in normal and malignant hematopoietic cells.

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EXPERIMENTAL

Materials. 5HmdUrd and proteinase K were purchased from the Sigma Chemical Co. (St. Louis, Missouri); phytohemagglutinin (PHA) was from Difco (Detroit, Michigan); radioactive chemicals were from Amersham International PLC (Amersham, U.K.); bases, nucleosides, and nucleotides were from the Calbiochem-Behring Corp. (La Jolla, California) and from Sigma; chromatography plates were from E. Merck (Darmstadt, F.R.G.); tissue culture media and antibiotics were from Gibco Europe Ltd. (Middlesex, U.K.); culture flasks were from A/S Nunc (Roskilde, Denmark).

Synthesis of [2-^{14}C]5HmdUrd. The synthesis of [2-^{14}C]5HmdUrd was performed as follows: In a 10 ml pear-shaped flask fitted with a reflux condenser and a nitrogen line bypass were combined 2.8 mg non-radiolabeled 2′-deoxyuridine (0.012 mmol), [2-^{14}C]-2′-deoxyuridine (2.35 MBq, 0.25 mg), paraformaldehyde (11 mg, 0.37 mmol CH_2O), tetrabutylammonium fluoride [0.05 ml 1 M solution in tetrahydrofuran (THF)] and 0.5 ml *N,N*-dimethylformamide (DMF). The solution was heated at 60 °C until a complete transformation to product had occurred (42 h) as shown by TLC assay (silica gel, eluted with 25 % methanol/$CHCl_3$). The solution was applied onto a 20×20 cm silica gel G-60 plate which was developed with 25 % methanol/$CHCl_3$. The UV-absorbing band containing the product was scraped off and the product was eluted with methanol (20 ml). The methanol solution was evaporated to dryness, and the residue was dissolved in water and centrifuged at 15 000 rpm. Lyophilization of the supernatant left a white powder which gave a single spot on TLC (25 % methanol/$CHCl_3$) corresponding to pure 5HmdUrd (R_f 0.42). From analysis of small aliquot, the product contained 850 kBq (overall yield 36 %). The specific radioactivity was 175 MBq/mmol.

Cells and media. The human leukemia cell lines (Table 1) were a generous gift from Professor Leif Andersson, Department of Pathology, University of Helsinki. The leukemic cells were maintained in 50 ml culture flasks in RPMI 1640 medium supplemented with 10 % fetal calf serum (20 % with HL-60 cells), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cultures did not contain mycoplasma when examined by staining with Hoechst Compound 33258. Human peripheral blood lymphocytes were isolated from healthy donors by density gradient centrifugation.[16] The culture methods have been described in more detail elsewhere.[17–19]

Cytotoxicity tests. All assays were performed on Cooke microtiter V plates (Sterilin Ltd., Middlesex, U.K.). The cultures were initiated with 2×10^4 cells in a volume of 200 μl/well. The cells were allowed to proliferate at 37 °C in humified, CO_2-controlled (5 %) atmosphere. The toxicity of 5HmdUrd was evaluated either by hemocytometric cell counting of viable cells,[20] or by incorporation of [U-^{14}C]-L-leucine for the final 4 h, after which the proteins were precipitated by 0.2 N PCA and collected on a glass fiber filter (Titertek Cell Harvester Filter, Flow Laboratories, Irvine, U.K.) using a multiple cell harvester (Cell Harvester D-001, Flow Laboratories). The radioactivity incorporated into proteins was measured in a scintillation spectrophotometer (LKB-Wallac, 81 000) with a counting efficiency of approximately 50 %.[19]

Incorporation studies. The incorporation of [2-^{14}C]5HmdUrd (specific activity 175 MBq/mmol), [methyl-^{14}C]thymidine (140 MBq/mmol), and [2-^{14}C]5-ethyl-2′-deoxyuridine

Table 1. Cell lines.

Cell lines	Original diagnosis	Ref.
BALL-1	Acute B-cell-leukemia	10
HL-60	Acute promyelocytic leukemia	11
JM	Acute T-cell leukemia	12
K-562	Chronic granulocytic leukemia in blast crisis	13
NALL-1	Null cell lymphoblastic leukemia	10
Raji	Burkitt's lymphoma	14
U-937	Histiocytic lymphoma	15

(133 MBq/mmol) were investigated by exposing exponentially growing human PHA-stimulated lymphocytes in 100 µl cultures for 60 min to 0.4 kBq of the compounds, after which DNA was isolated and hydrolyzed with formic acid: The cells were washed twice with ice cold phosphate-buffered saline (PBS) and the cell pellets were dissolved in 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 h at 37 °C. DNA and RNA were separated by phenol extractions, precipitated with ethanol and washed three times in order to remove phenol. RNA was hydrolyzed for 16 h at 37 °C with 0.3 N KOH. DNA was precipitated again with ethanol and traces of RNA bases were removed by three washes with 70 % ethanol. The isolated radioactive DNA was then hydrolyzed with formic acid and bases were separated with a two-dimensional chromatography on cellulose plates first with butanol–ammonia–water (86:4:10) and then with butanol–water (86:14). Marker molecules were localized under UV-light. The spots were scraped off and the radioactivity counted. Approximately 70 % of the DNA-bound radioactivity was recovered from the plates. The incorporation of [2-^{14}C]5HmdUrd to JM cells was assassed in a slightly modified way: Cells were exposed to 3 kBq (84 µM) of the compound in 200 µl subcultures for 60 min. In the third incorporation assay (Fig. 3) DNA was isolated according to a modified method of Schmidt at Thannhauser as described elsewhere.[18]

RESULTS

Toxicity studies. The influence of various initial cell concentrations on cell harvests was tested in 3-day cultures. The lowest concentration tested, *i.e.*, 10^5 cells/ml or 2×10^4 cells/well, resulted in the most vigorous growth (Fig. 1). The population doubling time (T_d) can be calculated from the equation;[21]

$$N_t=N_o\times2^{t/T_d}$$

where N_t is the cell number after culture, N_o is the initial cell number, and t is the duration of the culture. The following T_d values were obtained in 0–3 day cultures; BALL-1 35.4 h, HL-60 50.4 h, JM 29.7 h, K-562 39.8 h, NALL-1 38.1 h, Raji 26.1 h, and U-937 31.4 h (calculated from the data shown in Fig. 1). As illustrated in Fig. 1, the growth slowed down

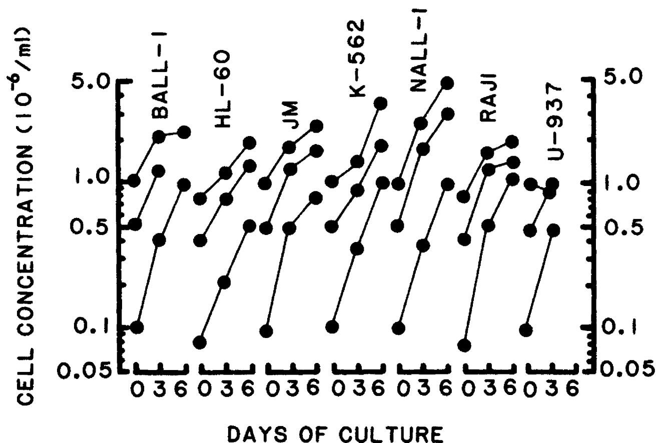


Fig. 1. Proliferation of established human leukemia cell lines in microplate cultures. Each point is an average cell number of two cultures. Living cells were counted hemocytometrically using trypan blue exclusion of viable cells.

Table 2. Toxicity of 5HmdUrd against normal and malignant hematopoietic cells in 3-day culture.

5HmdUrd/M	[U-^{14}C]-L-leucine incorporation/% of control±SD[a]								
	BALL-1	HL-60	JM	K-562	NALL-1	Raji	U-937	PHA-lymphocytes I	PHA-lymphocytes II
0	100±20	100±15	100±10	100±11	100±18	100±41	100±12	100±7	100±36
10^{-6}	91±10	90±27	101±3	101±14	79±14	83±27	106±23	97±10	75±4
10^{-5}	95±8	85±7	88±6	85±9	64±16	66±24	97±14	102±10	83±22
10^{-4}	34±3	10±3	9±1	8±9	2±1	9±2	21±5	55±10	41±12
10^{-3}	9±5	2±1	8±1	2±1	1±1	8±2	2±3	22±10	15±3
ID_{50} (M)[b]	5.8×10^{-5}	2.9×10^{-5}	3.1×10^{-5}	2.9×10^{-5}	1.7×10^{-5}	1.9×10^{-5}	4.1×10^{-5}	8.0×10^{-5}	6.0×10^{-5}

[a] The cultures were performed in quadruplicate. [U-^{14}C]-L-leucine (96 GBq/mmol; 0.75 kBq/well) was present for the four final h of the 3-day culture. [b] ID_{50}, the concentration of 5HmdUrd causing 50 % reduction in leucine incorporation, was calculated from the data presented in this Table.

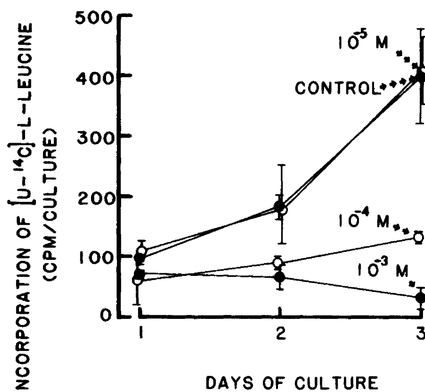


Fig. 2. Cytotoxic effect of 5HmdUrd during 3-day culture of BALL-1 cells. Each point is a mean (±SD) of four cultures.

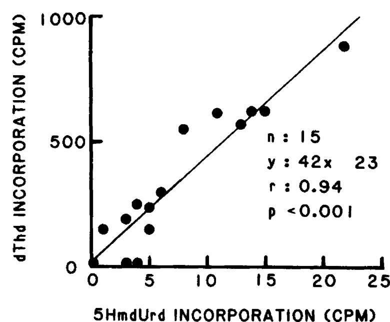


Fig. 3. Correlation of [methyl-^{14}C]thymidine and [2-^{14}C]-5HmdUrd incorporation into DNA of PHA-stimulated lymphocytes. The cells were exposed for 60 min in 1 ml subcultures to 0.4 kBq of [2-^{14}C]5HmdUrd (specific actitity 175 MBq/mmol) or [methyl-^{14}C]thymidine (140 MBq/mmol), after which the DNA was isolated and the radioactivity counted. Linear regression was calculated according to the least-squares method after subtracting the background activity (20 cpm).

Table 3. Radioactivity of DNA bases of human PHA-stimulated lymphocytes after exposure to [^{14}C]-labeled deoxyuridine analogues. The background (not subtracted from these figures) was 20±2 cpm.

Bases	Radioactivity/cpm per 5×10^7 cells		
	[2-^{14}C]5HmdUrd	[methyl-^{14}C]dThd[a]	[2-^{14}C]5EtdUrd[a]
Gua	50	33	26
Cyt	20	20	18
5HmUra	70	18	19
Ura	20	15	20
Ade	21	22	21
Thy	54	393	40
5EtUra	19	10	406

[a] Data from Ref. 19.

after the third day of culture. The higher initial cell counts, *i.e.*, 5×10^5 cells/ml and 10^6 cells/ml resulted in slower proliferation of cells independent of the cell types studied (Fig. 1).

The results of the toxicity assays with 7 leukemia cell lines and with normal human peripheral blood PHA-stimulated lymphocytes from two different donors are given in Table 2. Fifty percent inhibition in protein synthesis was observed with 5HmdUrd concentrations varying from 1.7 to 5.8×10^{-5} M. PHA-stimulated lymphocytes were the most resistant cells in this assay (Table 2).

The toxicity of even the highest concentration of 5HmdUrd was not readily observable during the first 24 h of culture, but it became more obvious after two and particularly after three days of culture (Fig. 2).

Incorporation of [2-^{14}C]5HmdUrd into DNA. The rate of incorporation of [2-^{14}C]5HmdUrd-derived radioactivity into DNA of human PHA-stimulated lymphocytes was significantly lower than that of [methyl-^{14}C]thymidine. The ratio of [methyl-^{14}C]thymidine to [2-^{14}C]5HmdUrd incorporation remained constant, *i.e.*, 42/1, during PHA-stimulation of human lymphocytes, as shown in Fig. 3. Most of the [2-^{14}C]5HmdUrd-derived radioactivity in the DNA hydrolysate of the PHA-stimulated lymphocytes co-migrated with 5-hydroxymethyluracil, although some activity was also observed in the thymine-spot in two-dimensional TLC (Table 3).

An analysis of radioactivity in individual DNA bases obtained from JM cells exposed for 60 min to 84 μM [2-^{14}C]5HmdUrd revealed that 50−80 % of the total radioactivity co-migrated with 5-hydroxymethyluracil and 10−20 % with thymine on TLC on cellulose plates (Fig. 4).

DISCUSSION

The toxicity of 5HmdUrd has been demonstrated in several organisms. However, the experiments performed so far have revealed resistance of human cancer against this compound; even a 10^{-4} M concentration of 5HmdUrd was not toxic to HeLa cells.[2] The present results show that 5HmdUrd is toxic to different types of human leukemia cells. Of possible relevance from a chemotherapeutic point of view is our observation that normal immunocompetent human cells, *i.e.*, PHA-stimulated lymphocytes, were less susceptible to 5HmdUrd than the leukemic cells.

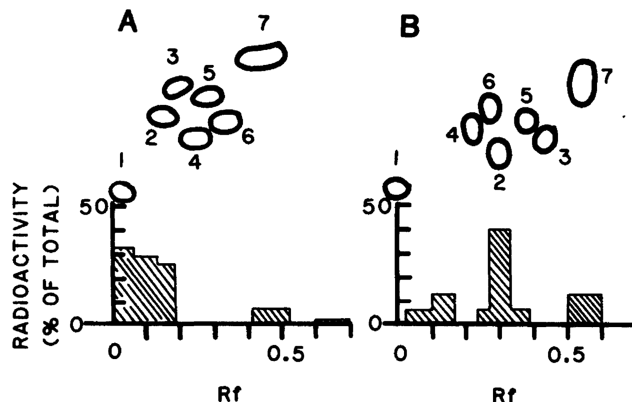
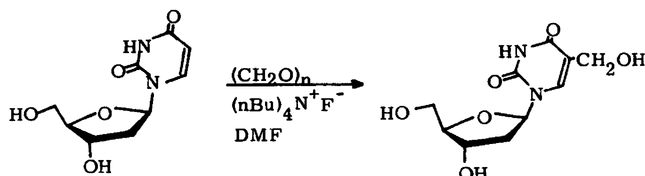


Fig. 4. Chromatography of DNA hydrolysate of [2-^{14}C]-5HmdUrd-exposed JM cells. The cultures were initiated with 2×10^4 JM cells and exposed for 60 min to 84 μM (3 kBq) [2-^{14}C]5HmdUrd, after which the DNA was isolated and hydrolyzed to its constituent bases with formic acid. The bases were chromatographed on cellulose plates with indicated markers; A. development with butanol–ammonia–water (86:4:10), B. butanol–water (86:14). Markers: 1 guanine (application area), 2 5-hydoxymethyluracil, 3 uracil, 4 cytosine, 5 adenine, 6 5-methylcytosine, and 7 thymine.

5HmdUrd is a close structural analogue of thymidine and it is conceivable that it does not have any immediate effect on protein synthesis. Our cytotoxicity assay based on leucine incorporation supports this view, since the toxicity of the compound became obvious only after 24 h exposure. Thus, it is evident that 5HmdUrd requires active cell proliferation and cellular incorporation in order to express its cytotoxicity.

A prerequisite for our metabolic studies with 5HmdUrd was the synthesis of a radioactive compound. We considered a [^{14}C]-labeled derivative to be superior to a [^{3}H]-labeled one because tritium is easily exchangeable with hydrogen. The preparation of [2-^{14}C]5HmdUrd has not been previously described, although that of [^{3}H]5HmdUrd has been reported.[22] The tritium-labeled compound can be prepared by ^{3}H exchange with unlabeled 5HmdUrd, which in turn can be prepared either from 5-bromomethyl-2′-deoxy-3′,5′-di-*O*-acetylthymidine[2] or by direct reaction of 2′-deoxyuridine with formaldehyde in aqueous hydroxide.[23] The former route is somewhat circuitous, requiring four steps from thymidine. One-step condensation with formaldehyde typically works well with uridine derivatives,[24] but gives poor results with 2′-deoxyuridine. We have found that the hydroxymethylation of 2′-deoxyuridine is improved considerably, and can be driven to completion, if tetrabutylammonium fluoride is used as the base in either pyridine or *N,N*-dimethylformamide. The reaction (Scheme 1) proceeds successfully on a scale ranging from less than one mg to greater than one g of 2′-deoxyuridine.



According to our experiments with [2-^{14}C]5HmdUrd most of the radioactivity incorporated into DNA was in 5-hydroxymethyluracil. This observation is in accordance with the results obtained by Matthes and coworkers with [^{3}H]5HmdUrd[22]. However, unlike Matthes' group, we did not observe any major radioactive DNA-associated component in the DNA hydrolysate: According to their report an unidentified compound comprised approximately 50 % of the DNA-associated radioactivity. On the other hand, we found some [2-^{14}C]5HmdUrd-derived radioactivity co-migrating with thymine while some remained close to the application area. A minor [2-^{14}C]thymidine contamination was observed in our [2-^{14}C]5HmdUrd preparation, comprising 0.6 % of the total radioactivity. This alone might have been responsible for the incorporation of radioactivity into thymidine.

The results of this investigation revealed that even a minor incorporation of 5HmdUrd into DNA was associated with profound cytotoxicity in several types of human leukemia cell lines. There is some evidence that 5-hydroxymethyluracil may be enzymatically removed – like uracil – when present in cellular DNA.[9] Such a repair of 5HmdUrd-induced DNA lesions might be associated with the toxicity of the compound. It can be speculated that an effective excision of newly-incorporated 5-hydroxymethyluracil from DNA is responsible for the low net incorporation rate observed in this work. The situation seems to be analogous to incorporation and excision of uracil.[9] Furthermore, radiation-induced formation of 5-hydroxymethyluracil from thymine in DNA may be responsible for crosslinking of DNA to chromatin proteins.[8] It remains to be clarified whether these mechanisms operate in human leukemic cells exposed to 5HmdUrd.

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