

## 5-Ethyl-2'-Deoxyuridine. Cytotoxicity and DNA Incorporation Demonstrated with Human Leukemic Cells and PHA-Stimulated Lymphocytes *in vitro*

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5-Ethyl-2'-deoxyuridine (5EtdUrd) is a biologically active thymidine analogue. The cytotoxicity of 5EtdUrd was investigated with seven established human leukemia cell lines as well as with human peripheral blood PHA-stimulated lymphocytes. All types of leukemia cells were susceptible to the toxicity of 5EtdUrd as assayed with a [U-<sup>14</sup>C]-L-leucine incorporation system developed for this study. A 50 % inhibition of leucine incorporation in 3-day cultures was induced by 1.3-3.8  $\mu$ M 5EtdUrd with leukemic cells, but the concentration required to induce similar inhibition with PHA-stimulated lymphocytes was approximately 100-fold. The toxicity of 5EtdUrd seemed to require active DNA synthesis, since the inhibition of leucine incorporation became obvious only after the first 24 hours of culture.

The DNA incorporation studies were based on a new isotopically labeled 5EtdUrd derivative, [2-<sup>14</sup>C]5EtdUrd, synthesized for this study in our laboratory. It was demonstrated for the first time that most of the radioactivity derived from [2-<sup>14</sup>C]5EtdUrd in DNA was in 5-ethyluracil.

5EtdUrd has a powerful antileukemic potency *in vitro*. Its effects against human leukemia *in vivo* remain to be tested.

5-Ethyl-2'-deoxyuridine (5EtdUrd) is a biologically active compound which readily incorporates into viral<sup>1-3</sup> (see also Ref. 4), bacterial,<sup>5</sup> and mammalian cell DNA.<sup>6-8</sup> The compound inhibits *in vitro* proliferation of the above-mentioned organisms and cells with the exception of human fibroblasts.<sup>6</sup> In preliminary work we have observed that 5EtdUrd exerts powerful antileukemic toxicity *in vitro*.<sup>9</sup> In this paper we report the results of cytotoxicity tests performed with 5EtdUrd against seven selected human leukemic cell lines as well as against normal human peripheral blood PHA-stimulated lymphocytes. We describe here a new synthesis method for [2-<sup>14</sup>C]5EtdUrd and show that 5EtdUrd is readily incorporated into the DNA of malignant and benign hematopoietic cells as 5-ethyluracil, although considerable differences in cytotoxicities between normal and malignant lymphocytes were observed.

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## MATERIALS AND METHODS

**Materials.** RPMI 1640 culture medium, fetal calf serum, L-glutamine, penicillin and streptomycin were obtained from Gibco Ltd. (Middlesex, England); Hoechst Compound 33258 was from Calbiochem-Behring Corp. (La Jolla, California); phytohemagglutinin was from Difco (Detroit, Michigan); proteinase K was from Sigma Chemical Co. (St. Louis, Missouri); bases and nucleosides were from Calbiochem-Behring Corp. and from Sigma Chemical Co.; Insta-Gel II scintillation liquid was from Packard Instrument Company Inc. (Dovers Grove, Illinois).

**Synthesis of [2-<sup>14</sup>C]5-Ethyl-2'-deoxyuridine.** The synthesis was performed (D.E.B.) as follows: In a 10 ml pear-shaped flask fitted with a magnetic stirring bar, a reflux condenser and a nitrogen line bypass, were combined 2.8 mg 2'-deoxyuridine (0.012 mmol), mercury(II)-acetate (7.5 mg, 0.0235 mmol), [2-<sup>14</sup>C]-2'-deoxyuridine (1.73 MBq, specific activity 2150 MBq/mmol, 0.184 mg; The Radiochemical Centre, Amersham, England), and distilled water (1.20 ml). The mixture was heated at 50 °C for 12 h. The cooled reaction mixture was lyophilized, a solution of 0.1 M Li<sub>2</sub>PdCl<sub>4</sub> in methanol added (260 μl, 0.026 mmol), and the mixture diluted to 1 ml with methanol. The flask was fixed with plasticine clay onto the bottom of a 500 ml Parr bottle and stirred magnetically under an ethene atmosphere (62 kPa) for 12 h using an apparatus similar to that described by Barefield.<sup>10</sup> The flask was evacuated *via* a water aspirator, 6.8 mg 10 % Pd/C suspended in 0.5 ml methanol added, and the apparatus pressurized with hydrogen (76 kPa). After 12 h the reaction mixture showed a single spot on TLC (silica gel G-60, 10 % methanol/CHCl<sub>3</sub>) at R<sub>f</sub> 0.25, corresponding to 5EtdUrd.<sup>11</sup>

The whole methanol solution was streaked onto a 20×20 cm silica gel G-60 plate and eluted with 15 % methanol/CHCl<sub>3</sub>. The UV absorbing band containing the 5EtdUrd was scraped off and the silica gel washed with a total of 15 ml methanol. Evaporation of the methanol gave a solid, which was then dissolved in 1.0 ml distilled water and centrifuged at 15 000 rpm. Lyophilization of the supernatant gave the product as an off-white solid, which by TLC analysis was pure 5EtdUrd. From analysis of a small aliquot, 0.334×10<sup>8</sup> dpm were counted, which corresponds to 555 kBq and an overall yield of 32 %. The specific activity was 130 MBq/mmol.

**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 cell lines were as follows: BALL-1 is an acute lymphoblastic leukemia with B cell differentiation.<sup>12</sup> HL-60 is an acute promyelocytic leukemia line.<sup>13</sup> JM is an acute T cell leukemia line.<sup>14</sup> K-562 is derived from a case of chronic granulocytic leukemia in blast crisis.<sup>15</sup> This line has a Philadelphia chromosome and it possesses erythroid and myeloid features. NALL-1 is an acute lymphoblastic leukemia cell line with neither B nor T cell differentiation.<sup>12</sup> Raji represents a cell line derived from Burkitt's lymphoma.<sup>16</sup> U-937 is a histiocytic lymphoma cell line.<sup>17</sup> Human peripheral blood lymphocytes were isolated from healthy donors by density gradient centrifugation.<sup>18</sup>

The leukemia cells were maintained in 260 ml culture flasks (A/S Nunc, Roskilde, Denmark) in RPMI 1640 supplemented with 10 % fetal calf serum (20 % with HL-60), 2 mM L-glutamine, 100 U penicillin per ml, and 100 μg streptomycin per ml. In maintaining cultures new medium was added twice a week. The cultures did not contain Mycoplasma at the detection level reached by staining with Hoechst Compound 33258.<sup>19</sup> Peripheral blood lymphocytes were grown in similar conditions in the presence of phytohemagglutinin (7 μg/ml).

**Cytotoxicity tests.** All assays were performed on Cooke Microtiter V plates (Sterilin Ltd., Middlesex, England). The tests were performed under optimal growth conditions as described in detail elsewhere.<sup>20</sup> The given amount of 5EtdUrd was placed in the culture wells together with 200  $\mu$ l of cell suspension containing  $2 \times 10^4$  leukemic cells or  $10^5$  peripheral blood mononuclear cells. The cells were allowed to proliferate for 68–74 h at 37 °C in a humidified, CO<sub>2</sub>-controlled (5 %) atmosphere. At the end of the incubation, living cells were counted using their ability to exclude trypan blue stain in a hemocytometer,<sup>21</sup> or the cells were exposed for the four final hours to 0.74 kBq of [U-<sup>14</sup>C]-L-leucine (specific activity 96 MBq/mmol) and harvested by a multiple cell harvester (Cell Harvester D-001, Flow Laboratories Ltd., U.K.). Firstly, macromolecules were precipitated by adding 8  $\mu$ l of 5 M perchloric acid to each culture well. The microplates were incubated for 15 min in an ice-bath. The contents of the microplate wells were collected on glass fiber filter (Titertek Cell Harvester Filters, Flow Laboratories) and rinsed for 40 s with 0.2 M perchloric acid. Radioactivities were measured in a scintillation spectrophotometer (LKB-Wallac 81 000) with a counting efficiency of approximately 50 %.

**Incorporation studies.** The incorporation studies were performed by exposing human PHA-stimulated lymphocytes for one h or BALL-1 and Raji cells for 72 h to radioactive 5EtdUrd. Individual cellular fractions were collected making use of a modified Schmidt-Thannhauser method as follows: the cells were first washed with PBS (phosphate-buffered saline, pH 7.4). They were then dissolved in 666  $\mu$ l of water containing 150  $\mu$ g of highly polymerized carrier DNA. The tubes were incubated in an ice-bath and 333  $\mu$ l of 0.6 M 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 (10 000 $\times$ g, 2 min). The precipitates were washed once with 1 ml of 0.2 M perchloric acid. They were then dissolved in 0.5 ml of 0.3 M KOH and RNA was hydrolyzed for 60 min at 37 °C.<sup>22</sup> The hydrolysate was acidified by adding perchloric acid to a final concentration of 0.2 M, and DNA and protein were precipitated, dissolved in 20  $\mu$ l of 1 M NaOH and 1 ml of water. The DNA-containing fraction was isolated by hydrolysis with 1 M perchloric acid at 80 °C. This treatment was repeated. The radioactivity in the various cellular compartments was counted in a scintillation spectrophotometer.

The analysis of radioactivity of individual bases of DNA in human PHA-stimulated lymphocytes and JM cells was performed after exposure to [2-<sup>14</sup>C]5EtdUrd as follows: human PHA-stimulated lymphocytes were concentrated by centrifugation and exposed in 100  $\mu$ l of culture medium to 3.7 kBq of [2-<sup>14</sup>C]5EtdUrd (specific activity 133 MBq/mmol) or 3.7 kBq of [methyl-<sup>14</sup>C]thymidine (specific activity 141 MBq/mmol). The cells were washed twice with ice-cold PBS and the cell pellets were dissolved in 50  $\mu$ l 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 h at 37 °C. The nucleic acids were separated by four successive phenol extractions. The nucleic acids were then precipitated with ethanol and washed three times in order to remove phenol. RNA was hydrolyzed for 16 h at 37 °C with 0.3 M KOH. DNA was precipitated again with 70 % ethanol. DNA was hydrolyzed with formic acid, 30 min at 175 °C and the bases were separated chromatographically on cellulose plates (E. Merck): chromatography was carried out at room temperature (20–24 °C) in a glass chromatography tank with no pretreatment. 1  $\mu$ l of each base (see below) from a 50 mM water solution stock, together with the sample aliquot, was applied to the lower left corner of the plate. The first ascending development was performed to 18–19 cm from the bottom with butanol–ammonia–water (86:4:10). The plate was dried with a blow drier and the development was repeated. A second ascending development in a 90

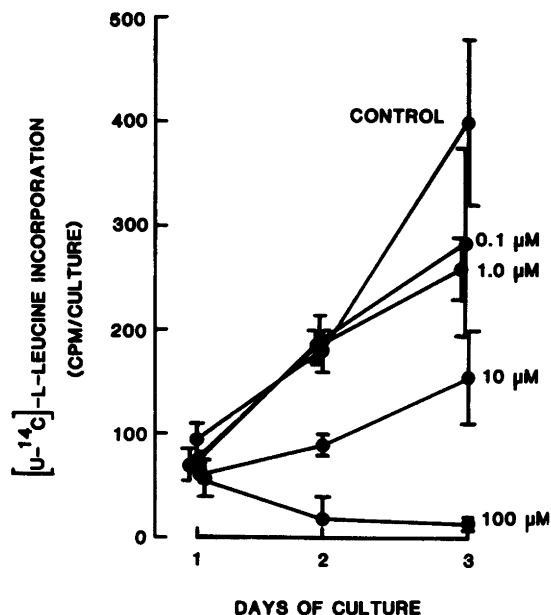


Fig. 1. Toxicity of 5EtdUrd studied daily in BALL-1 cell cultures. The cells were grown in microplate cultures initially containing 0 to 100  $\mu\text{M}$  5EtdUrd. Radioactive leucine was added for the four final hours, after which the proteins were precipitated with perchloric acid. The precipitates were collected by a multiple cell harvester onto glass fiber filters and radioactivity was counted.

degree direction was performed with butanol–water (86:14). This step was also repeated. The marker molecules were localized under UV light, the spots were cut off and the radioactivity counted. Full separation of the following markers was obtained: adenine, cytosine, 5-ethyluracil, guanine, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5-methylcytosine, thymine and uracil.

## RESULTS

**Cytotoxicity tests.** 5EtdUrd was toxic to all leukemic cell types when present at a 10  $\mu\text{M}$  concentration. An example of the manifestation of this toxic effect of 5EtdUrd is demonstrated in Fig. 1; the reduction of  $[\text{U-}^{14}\text{C}]\text{-L-leucine}$  incorporation was not obvious over the first 24 h of culture but it manifested thereafter in a dose-dependent fashion as shown with BALL-1 cells in Fig. 1. Similar time/ $[\text{U-}^{14}\text{C}]\text{-L-leucine}$  incorporation curves were obtained with all other leukemic cell lines assayed in this work (results not shown). The cytotoxicity of 5EtdUrd appeared to be independent of the leukemic cell type tested. A 60% or higher reduction in  $[\text{U-}^{14}\text{C}]\text{-L-leucine}$  incorporation was achieved with 10  $\mu\text{M}$  5EtdUrd, as shown in Table 1. The corresponding growth inhibition by 100  $\mu\text{M}$  5EtdUrd was 93–100%. The inhibition of protein synthesis by 5EtdUrd in normal human peripheral blood PHA-stimulated lymphocytes was much weaker than that in leukemic cells (Table 1).

**Incorporation of  $[\text{2-}^{14}\text{C}]\text{5EtdUrd}$  into DNA.**  $[\text{2-}^{14}\text{C}]\text{5EtdUrd}$  was incorporated into DNA of human peripheral blood PHA-stimulated lymphocytes, as illustrated in Fig. 2. The ratio of 5EtdUrd- to thymidine-incorporation was approximately 0.4 in spite of the great variation of proliferative activity of the cells at different times after the initiation of the cultures (Fig. 2). The radioactivity derived from  $[\text{2-}^{14}\text{C}]\text{5EtdUrd}$  was present in 5-ethyluracil and none was detected in guanine, adenine, cytosine or 5-hydroxymethyluracil, when purified DNA was hydrolyzed with formic acid and the bases analyzed by a two-dimensional thin-layer

Table 1. Cytotoxicity of 5EtdUrd in 3-day cultures.

[5EtdUrd]/ $\mu\text{M}$	[U- <sup>14</sup> C]-L-leucine incorporation/% of control $\pm$ SD <sup>a</sup>									
	BALL-1	HL-60	JM	K-562	NALL-1	Raji	U-937	PHA-lymphocytes		
								I	II	
0	100 $\pm$ 20	100 $\pm$ 13	100 $\pm$ 10	100 $\pm$ 13	100 $\pm$ 12	100 $\pm$ 30	100 $\pm$ 4	100 $\pm$ 14	100 $\pm$ 27	
1	69 $\pm$ 4	76 $\pm$ 14	87 $\pm$ 14	59 $\pm$ 9	74 $\pm$ 13	55 $\pm$ 12	112 $\pm$ 11	92 $\pm$ 12	93 $\pm$ 20	
10	40 $\pm$ 10	6 $\pm$ 4	12 $\pm$ 5	13 $\pm$ 3	28 $\pm$ 13	10 $\pm$ 4	7 $\pm$ 12	69 $\pm$ 10	93 $\pm$ 15	
100	6 $\pm$ 2	0 $\pm$ 1	5 $\pm$ 4	7 $\pm$ 1	3 $\pm$ 3	4 $\pm$ 4	2 $\pm$ 3	46 $\pm$ 5	65 $\pm$ 4	
ID <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>	1.5	2.3	3.0	1.4	3.2	1.3	3.8	60	100	

<sup>a</sup> Four cultures were made. [U-<sup>14</sup>C]-L-leucine (0.74 kBq) was present for the final four hours of culture. <sup>b</sup> ID<sub>50</sub> is the concentration of 5EtdUrd causing a 50 % reduction in cell proliferation (leucine incorporation).

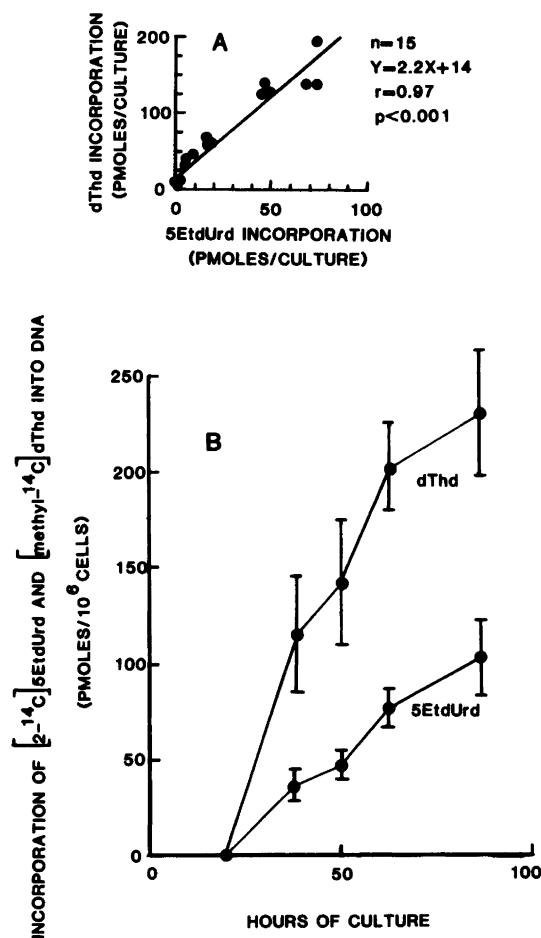


Fig. 2. Incorporation of  $[2-^{14}\text{C}]5\text{EtdUrd}$ - and  $[\text{methyl-}^{14}\text{C}]$ thymidine-derived radioactivity into the DNA of human peripheral blood PHA-stimulated lymphocytes. After various culture periods radioactive  $5\text{EtdUrd}$  and thymidine were added to separate 1 ml subcultures. After 60 min exposure the DNA was isolated by a modified Schmidt-Thannhauser procedure and the radioactivity was counted. (A) Correlation of thymidine (dThd)-derived incorporation analyzed by the least-squares regression method. (B) The same data as in (A) presented as progress of incorporation of  $5\text{EtdUrd}$ - and thymidine-derived radioactivity during PHA-stimulation. Each point represents the mean ( $\pm\text{SD}$ ) of three cultures.

chromatography. Some radioactivity, however, co-migrated with thymine (Table 2). Most of the radioactivity in isolated DNA from  $[2-^{14}\text{C}]5\text{EtdUrd}$ -exposed leukemic JM cells was also in 5-ethyluracil. This was demonstrated by two-dimensional thin-layer chromatography (results not shown), as for PHA-stimulated peripheral blood lymphocytes.

$[2-^{14}\text{C}]5\text{EtdUrd}$  incorporation was also demonstrated in 3-day cultures of human leukemic cell lines BALL-1 and Raji. Increasing concentrations of  $5\text{EtdUrd}$  tended to increase the proportion of radioactivity in the acid-soluble fraction, as illustrated in Table 3.

Table 2. Radioactivity in individual DNA-bases of human peripheral blood PHA-stimulated lymphocytes exposed for 60 min to [ $^{14}\text{C}$ ]-labeled 5EtdUrd and thymidine. The background activity (not subtracted) was  $20 \pm 3$  cpm.

Base	Radioactivity (cpm/ $5 \times 10^7$ cells)	
	[2- $^{14}\text{C}$ ]5EtdUrd	[methyl- $^{14}\text{C}$ ]thymidine
Guanine	26	33
Cytosine	20	18
5-Hydroxymethyluracil	20	18
Uracil	20	15
Adenine	21	22
Thymine	40	393
5-Ethyluracil	406	19

Table 3. Incorporation of [2- $^{14}\text{C}$ ]5EtdUrd in BALL-1 and Raji cells in 3-day cultures.

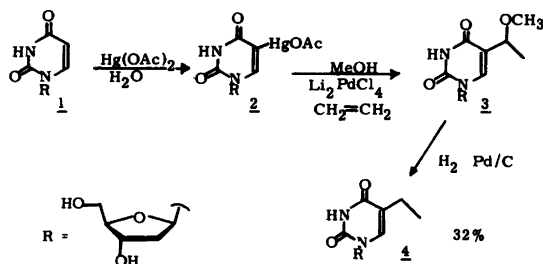
Fraction	Incorporation (pmol/ $10^6$ cells $\pm$ SD) <sup>a</sup>			
	BALL-1		Raji	
	5EtdUrd/25 $\mu\text{M}$	5EtdUrd/100 $\mu\text{M}$	5EtdUrd/25 $\mu\text{M}$	5EtdUrd/100 $\mu\text{M}$
Acid sol.	58 $\pm$ 13	1294 $\pm$ 529	780 $\pm$ 99	8704 $\pm$ 2206
DNA	574 $\pm$ 122	3823 $\pm$ 647	1436 $\pm$ 254	5815 $\pm$ 1700

<sup>a</sup> Three cultures were analyzed. Under identical culture conditions in microplates 25  $\mu\text{M}$  5EtdUrd decreased the cell harvest by 19 % in BALL-1 cell cultures and by 43 % in Raji cell cultures, and 100  $\mu\text{M}$  5EtdUrd decreased the harvest by 83 and by 82 % in BALL-1 cell and Raji cell cultures, respectively. 25  $\mu\text{M}$  5EtdUrd corresponded to 0.74 kBq and 200  $\mu\text{M}$  5EtdUrd to 2.96 kBq.

## DISCUSSION

A simplified procedure for preparation of 5EtdUrd on a microscale was developed for the purpose of obtaining radiolabeled material. Unlike the original large scale procedure described by Bergstrom and Ogawa,<sup>11</sup> the present procedure is done in a single flask without isolation of either intermediate. [2- $^{14}\text{C}$ ]-2'-deoxyuridine (1 in Scheme 1) and mercury(II)-acetate react in aqueous solution to give the 5-mercuri derivative, 2<sup>23</sup>. Reaction of 2 with  $\text{Li}_2\text{PdCl}_4$  and ethene in methanol gives 5-methoxyethyl-2'-deoxyuridine (3).<sup>11</sup> Nucleoside 3 was not isolated, but immediately subjected to hydrogenolysis ( $\text{H}_2$ , Pd/C) to remove the methoxy group, giving 5-ethyl-2'-deoxyuridine (4). This chemistry has been discussed elsewhere,<sup>11</sup> but was not previously accomplished in a singleflask procedure as described here.

The toxicity of 5EtdUrd has been demonstrated in various organisms such as viruses, bacteria and mammalian cells *in vitro*. The present investigation extends the data to most variants of human acute leukemia; 5EtdUrd was predictably toxic to all leukemia cell lines tested. The variability in the response between the lines was minimal. Namely, the doses inhibiting the protein synthesis of the culture by 50 % compared with the cultures without 5EtdUrd were within the range of 1.3 to 3.8  $\mu\text{M}$ . Comparable  $\text{ID}_{50}$  concentrations for mouse leukemia L1210<sup>24</sup> and human lymphoblasts<sup>25</sup> have been approximately ten times higher, or



Scheme 1.

33  $\mu\text{M}$  and 38  $\mu\text{M}$ , respectively. Considerable differences, however, can be observed in different sublines from the same origin, as in the case of Raji cells. The  $\text{ID}_{50}$  for Raji cells observed by De Clercq and coworkers<sup>25</sup> was 19.9  $\mu\text{g/ml}$  (78  $\mu\text{M}$ ) and our value was 1.3  $\mu\text{M}$ . The difference could be based on the thymidine kinase activity; low activities make cells more resistant to 5EtdUrd.<sup>25</sup>

The cytotoxicity of 5EtdUrd as measured by leucine incorporation was manifested after the first day of culture. This means that the compound does not immediately reduce the protein synthesis of leukemic cells. This experimental finding supports the predictable mechanism of action of 5EtdUrd as a thymidine antimetabolite.

A remarkable difference in the susceptibility to 5EtdUrd was demonstrated between normal and malignant T cells. Although the  $\text{ID}_{50}$  for JM was 3.0  $\mu\text{M}$ , the  $\text{ID}_{50}$  for human PHA-stimulated lymphocytes approached or even exceeded 100  $\mu\text{M}$ . The resistance of immunoreactive cells to 5EtdUrd has been reported in mice: 200 mg/kg/day given intraperitoneally for six days did not suppress the primary immunoresponse to an intravenous injection of sheep red cells.<sup>26</sup> This lack of effect, however, could possibly be accounted for by the rapid elimination of 5EtdUrd *in vivo* (*cf.* Ref. 7.). The relative resistance to 5EtdUrd of cultured human lymphocytes has also been observed when the cells were exposed to 100  $\mu\text{g}$  of 5EtdUrd per ml (390  $\mu\text{M}$ ) and no chromosomal aberrations were seen.<sup>6</sup>

It has been shown previously that 5EtdUrd-derived radioactivity is incorporated into the DNA of mammalian cells.<sup>6-8</sup> We have demonstrated for the first time that 5EtdUrd-derived radioactivity of isolated DNA-hydrolysate co-chromatographed mainly with 5-ethyluracil, although some radioactivity was also present in thymine. The possible thymidine contamination in the original [2-<sup>14</sup>C]5EtdUrd preparation was less than 1 %. It remains to be clarified whether 5EtdUrd can be metabolized to thymidine.

Experimental chemotherapy with 5EtdUrd has been hampered by the rapid elimination of the compound *in vivo*.<sup>7</sup> A catabolic enzyme, thymidine phosphorylase, is present in normal human tissues.<sup>27</sup> Nevertheless, virostatic concentrations of 5EtdUrd can be achieved in rodents<sup>28</sup> and according to our preliminary observations, the catabolism of 5EtdUrd in human blood is relatively slow (Vilpo, unpublished results). The final value of 5EtdUrd against human leukemia *in vivo*, alone or in combinations with other therapeutic agents, remains to be evaluated.

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