Biochemical Pharmacology, Vol. 35, No. 23, pp. 4211–4215, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

IN VITRO AND *IN VIVO* STUDIES OF A PROMISING ANTILEUKEMIC THYMIDINE ANALOGUE, 5-HYDROXYMETHYL-2′ DEOXYURIDINE

LEILA KAHILAINEN,* DONALD BERGSTROM,† LAURI KANGAS‡ and JUHANI A. VILPO*§

*Laboratory of Molecular Hematology, Department of Clinical Chemistry, University of Oulu, SF-90220 Oulu, Finland; †Department of Chemistry, University of North Dakota, Grand Forks, North Dakota 58202, U.S.A.; ‡Cancer Research Laboratory, Farmos Group Ltd., SF-20101 Turku, Finland

(*Received* 21 *October* 1985; *accepted* 16 *June* 1986)

Abstract—The toxicity and metabolism of a thymidine analogue, 5-hydroxymethyl-2′-deoxyuridine (5HmdUrd) were studied with human leukemia cells (HL-60) and with human platelets. 3×10^{-5} M 5HmdUrd caused a 50% inhibition in the proliferation of HL-60 cells. The compound was hydrolyzed to 5-hydroxymethyluracil (5HmUra) by the enzyme thymidine phosphorylase (EC 2.4.2.4) present in leukemia cells; this catabolic product was non-toxic. The catabolism of 5HmdUrd by human platelet thymidine phosphorylase could be inhibited by 6-aminothymine. The toxicity of 5HmdUrd was effectively reversed by deoxycytidine and 5HmdUrd increased the incorporation of deoxycytidine into dCTP and DNA several fold. The two latter phenomena are explicable in terms of a feedback action to ribonucleotide reductase, resulting in deoxycytidylate starvation, which is a known effect of excess thymidine. We report here also our preliminary observations that 5HmdUrd is active against mouse leukemia *in vivo*.

Several 5-carbon-modified deoxyuridine derivatives have shown activity in antiviral or anticancer chemotherapy. These include the antiviral agents 5-iodo-2′-deoxyuridine [1, 2], 5-(2-bromovinyl)-2′-deoxyuridine [3–6], and 5-trifluoromethyl-2′-deoxyuridine [7], as well as the anticancer agent 5-fluoro-2′-deoxyuridine and its precursor molecule 5-fluorouracil [8–10]. We have recently demonstrated that 5-hydroxymethyl-2′-deoxyuridine (5HmdUrd) is toxic against several types of human acute leukemia cell lines *in vitro* and the compound can be regarded as a potential antileukemia drug [11].

The present study was undertaken in order to characterize the metabolism of 5HmdUrd in human leukemia cell cultures. The results demonstrated that although the compound is accumulated into DNA at a relatively slow rate, its metabolic behavior in many respects resembles that of the normal analogue thymidine (dThd). The preliminary tests of 5HmdUrd against mouse leukemia L1210 *in vivo* are also reported.

MATERIALS AND METHODS

Chemicals. 5HmdUrd, 5HmUra, and other bases, nucleosides, and nucleotides were obtained from the Calbiochem-Behring Corp. (La Jolla, California) and from Sigma Chemical Co. (St. Louis, MO); highly polymerized DNA was from Sigma; tetrahydrouridine (THU) from the Drug Synthesis and Development Branch of the National Cancer Institute (U.S.A); chromatography plates from E. Merck (Darmstadt, F.R.G.); tissue culture media and antibiotics from Gibco Europe Ltd. (Middlesex, U.K.), and 6-aminothymine was prepared according to the procedure described by C. K. Cain *et al.* [12]. [2-^{14}C]5HmdUrd (sp. act. 4.75 mCi/mmole) was synthesized as described elsewhere [11]. Other radioactive chemicals were purchased from Amersham International PLC (Amersham, U.K.).

Cells. The human leukemia cell line HL-60 (an acute promyelocytic leukemia line [13]) was a generous gift from Professor Leif Andersson, Department of Pathology, University of Helsinki, Helsinki, Finland. The cells were maintained in 50 ml culture flasks (A/S Nunc, Roskilde, Denmark) in RPMI 1640 medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cultures did not contain mycoplasm at the detection level reached by staining with Hoechst compound 33258 [14].

Cytotoxicity studies. 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 with a given amount of 5HmdUrd or 5HmUra. The cells were allowed to proliferate for 24–72 hr at 37° in a humidified, CO_2-controlled (5%) atmosphere. The toxicity of the test compounds was evaluated by hemocytometric cell counting of viable cells [15].

Effects of deoxyribonucleosides on the toxicity of 5HmdUrd in 3-day cultures. The assays with other deoxyribonucleosides were performed on microplate cultures initiated with 2×10^4 cells in a volume of 200 μl/well together with given amounts of test compounds. The cells were allowed to proliferate for 72 hr at 37° in a humidified, CO_2-controlled (5%) atmosphere. For the final 24 hr, [U-^{14}C]-L-leucine (sp. act. 1.3 mCi/mmole, 0.5 μCi/ml) was added. After incubation the proteins were precipitated by 0.2 N PCA and collected on a glass fiber filter (Titertek Cell Harvester Filter, Flow Laboratories, Irvine,

§ Send all correspondence to: Dr. J. A. Vilpo, Department of Clinical Chemistry, Laboratory of Molecular Hematology, University of Oulu, SF-90220 Oulu, Finland.

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 this test system the handling of a large number of simultaneous cultures is possible. The incorporation of ^{14}C-leucine per cell remained constant (500 cpm/10^5 cells/hr) during the final 24 hr of culture and there was a good correlation between cell numbers and ^{14}C-leucine incorporation per well.

Separation of metabolites. We quantitated the rate of degradation of 5HmdUrd while tested for its toxicity by exposing exponentially growing cultures to [2-^{14}C]5HmdUrd. Culture medium without cells served as blank. After a given incubation period the cells were centrifuged (10,000 *g*; 2 min at +4°) and 2.5 vol. of ethanol were added to the supernatant. After 15 min in an ice bath the precipitated protein was removed by centrifugation (10,000 *g*; 15 min at +4°). 5HmdUrd (R_f 0.82) and the corresponding base (5HmUra, R_f 0.77) were separated using PEI-cellulose thin layer chromatography and 0.4 M LiCl for developing. The marker molecules were localized under u.v. light, the spots were cut out and the radioactivity counted in a scintillation spectrophotometer.

Degradation of 5HmdUrd, dThd, and Urd was examined in order to clarify the substrate spectrum of hydrolysis by exposing HL-60 cell cultures which were initiated with 2×10^5 cells per well, to [2-^{14}C]-5HmdUrd (0.2 μCi/ml or 42 μM), [methyl-^{3}H]dThd (5 μCi/ml or 30 μM), and [5-^{3}H]Urd (5 μCi/ml or 30 μM) for 30 min and for 4 hr. After incubation the base and the nucleoside were quantitated as described above.

The catabolism of 5HmdUrd and dThd by human peripheral blood platelets was studied with and without a specific inhibitor of thymidine phosphorylase, 6-aminothymine [16, 17]. Human peripheral blood platelets were isolated from healthy donors as follows: blood was drawn into lithium-heparinized vacuum tubes, white cells and red cells were separated by centrifugation (175 *g*; 10 min), and the platelets were obtained from the supernatant platelet-rich plasma. Two different platelet concentrations (in RPMI 1640 medium supplemented with 10% fetal calf serum) were used. Platelets were exposed to [2-^{14}C]5HmdUrd and [methyl-^{3}H]dThd with and without 10^{-4} M 6-aminothymine for 60 min at 37°. The bases and the nucleosides were then separated as for HL-60 cells.

Incorporation studies. The immediate effects of 5HmdUrd on the incorporation of dCyd into HL-60 cells was investigated with the potent cytidine deaminase inhibitor, THU [18]. Aliquots of 100 μl (200,000 cells) were placed in culture tubes containing appropriate amounts of 5HmdUrd, 1 μCi of [5-^{3}H]dCyd (22 Ci/mmole, 20 μCi/ml) and 10^{-4} M THU. After 15 min at 37° the cells were washed three times with ice-cold phosphate buffered saline (PBS). The cells were then dissolved in 600 μl of water and 50 μl of highly polymerized carrier DNA (2 mg/ml). The tubes were incubated in an ice bath and 333 μl of 0.6 N PCA 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 *g*; 2 min). The precipitates were then dissolved in 0.5 ml of 0.3 N KOH and RNA was hydrolyzed for 60 min at 37° [19]. The hydrolysate was acidified with PCA to a final concentration of 0.2 N and DNA together with protein was precipitated. After centrifugation (10,000 *g*; 2 min) the supernatant was rejected and the precipitate washed once with 0.5 ml of 0.2 N PCA. The DNA precipitate was then dissolved in 20 μl of 1 N NaOH and 1 ml of water, and the radioactivity was measured.

The methanol-soluble radioactive dCyd metabolites were isolated from washed cells by two extractions with 60% methanol at 30° (2×10 min). The extracts were combined and centrifuged at 10,000 *g* for 15 min. The supernatant was used as the intracellular metabolite fraction. Methanol extract (5 μl) and 1 μl of the marker solution containing known amounts of Cyt, dCyd, dCMP, dCDP, and dCTP were spotted on PEI-cellulose plates. The plates were developed with 0.4 M LiCl and dried. The u.v.-absorbing spots were marked, the plates were cut in 1–2 × 1–2 cm pieces and the radioactivity counted.

Animal experiments. The L1210 leukemic cells were maintained in the laboratory by weekly i.p. life transplant of 10^6 cells diluted in 0.1 ml saline in DBA/2 female mice. The drug was dissolved in saline and given i.p. starting one day after the tumor implantation.

RESULTS

Toxicity of 5HmdUrd and 5HmUra in vitro

The toxicity of 5HmdUrd and the corresponding base, 5HmUra, was analyzed in 3-day cultures of HL-60 cells. The dose-dependent toxicity of 5HmdUrd was demonstrable only after the first 24 hr of culture, but the corresponding doses of 5HmUra were not toxic (Fig. 1).

Catabolism of 5HmdUrd

The relevance of the experiment reported above became obvious when the catabolism of 5HmdUrd was investigated. Namely, we observed that the compound was readily catabolized to the corresponding base by the leukemia cells in the culture. The reaction reached completion earlier in those cultures containing lower initial concentrations of 5HmdUrd (Table 1).

We assumed that pyrimidine nucleoside phosphorylase was the enzyme responsible for the catabolism of 5HmdUrd. The substrate specificity of this enzyme was studied in some detail. We demonstrated that the enzyme present in intact leukemia cell cultures readily hydrolyzed dThd and to a lesser extent 5HmdUrd, but very little, if any Urd (Fig. 2). Moreover, we studied the catabolism of 5HmdUrd, utilizing human blood platelets as a source of thymidine phosphorylase [16, 17, 20]. The catabolism of 5HmdUrd was significantly slower than that of dThd (Fig. 3). 6-Aminothymine caused 31.8% and 38.5% inhibition in dThd hydrolysis and 50% and 70% inhibition in 5HmdUrd hydrolysis, respectively, depending on the platelet concentration (Fig. 3).

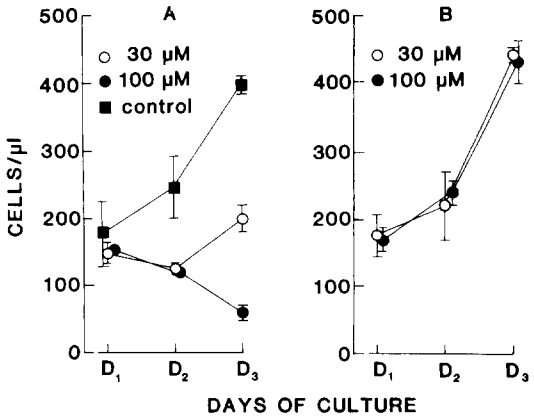

Fig. 1. Toxicity of 5-hydroxymethyl-2′-deoxyuridine (A) and 5-hydroxymethyluracil (B) against HL-60 cells *in vitro*. Each point is the mean (±SD) of triplicate cultures. The initial cell concentration was 100/μl.

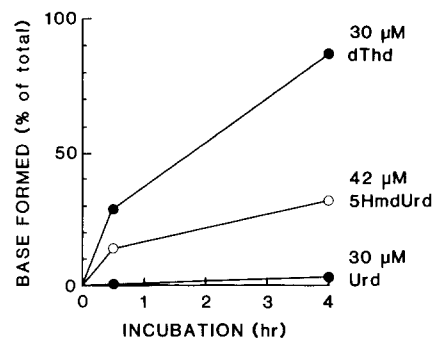

Fig. 2. Catabolism of thymidine (dThd), 5-hydroxymethyl-2′-deoxyuridine (5HmdUrd) and uridine (Urd) to corresponding bases by intact HL-60 cells. The incubation was performed at 37° with 2×10^5 cells in a volume of 200 μl. Each point represents the average of two determinations.

Effect of other deoxyribonucleosides on the toxicity of 5HmdUrd

The biochemical mode of 5HmdUrd action was further investigated by treating the leukemia cell cultures with the compound together with other deoxyribonucleosides. The toxicity of 5HmdUrd, as assessed by [^{14}C]leucine incorporation in 3-day cultures of HL-60 cells, was completely reversed by dCyd (Table 2). Partial reversal of toxicity was also attained with 10^{-4} M dThd, but the action of 10^{-3} M dThd could not be conclusively tested because of the toxicity of dThd (Table 2). No significant effects of other deoxyribonucleosides on the toxicity of 5HmdUrd were seen.

Effect of 5HmdUrd on the cellular incorporation of [^{3}H]dCyd

5HmdUrd induced a concentration-dependent enhancement in the incorporation of radioactivity from [5-^{3}H]dCyd into DNA and dCTP, and to a lesser extent into dCDP (Table 3). Even a little stronger effect was achieved with an equimolar concentration of dThd (Table 3).

Toxicity of 5HmdUrd against L1210 leukemia in vivo

The pilot experiment demonstrated that 5HmdUrd may prolong the survival of mice with L1210 leukemia. The survival times of four mice given 50 mg/kg i.p. per day for five days were 53, 21, 20 and 22 days, whereas the control animals survived for 12, 11, 11 and 12 days.

Table 1. Hydrolysis of [2-^{14}C]5HmdUrd to [2-^{14}C]5HmUra in HL-60 cell culture

	Base formed (% of total)*	
Days	5HmdUrd 30 μM	5HmdUrd 100 μM
0	0	0
1	41.0	21.5
2	88.0	66.0
3	93.5	95.0

* Average of two determinations.

DISCUSSION

The biological activity of 5HmdUrd against non-human and human cells has been known for some time [21–23]. However, systematic screening against different types of human hematopoietic malignomas has been done only recently in our laboratory; 5HmdUrd had a predictable toxicity against several human acute leukemia cell lines *in vitro* [11]. Furthermore, according to preliminary observations, the compound is also active against mouse leukemia L1210 *in vivo*.

We demonstrated in this study that the toxicity of 5HmdUrd became obvious only after the first 24 hr of culture, indicating that there is no immediate cytoxicity. The finding is consistent with the anti-

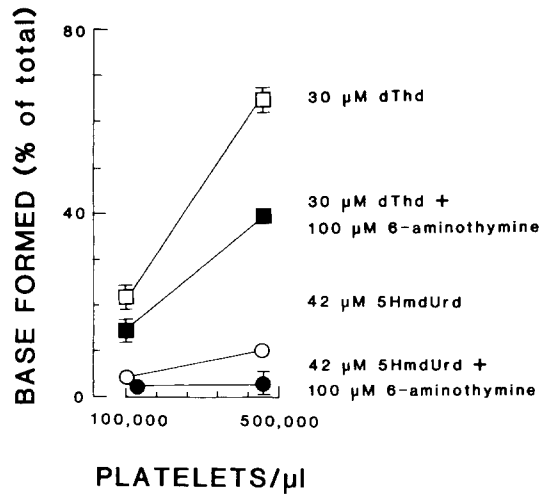

Fig. 3. Effect of 6-aminothymine on the catabolism of thymidine (dThd) and 5-hydroxymethyl-2′-deoxyuridine (5HmdUrd) by human platelets. The incubation was 60 min at 37° in a volume of 200 μl. Each point represents the mean of 3 determinations (±SD).

Table 2. Influence of 5HmdUrd alone and in various combinations with other nucleosides in the incorporation of [^{14}C]leucine into HL-60 cells in 3-day cultures

5HmdUrd (μM)	Additional treatment	[U-^{14}C]-L-leucine incorporation (cpm/well ± SD)*
Experiment I		
None	None	12,713 ± 256
100	None	6,484 ± 373
None	dCyd (100 μM)	13,230 ± 341
100	dCyd (100 μM)	9,516 ± 605
None	dThd (100 μM)	11,787 ± 441
100	dThd (100 μM)	10,685 ± 375
None	dUrd (100 μM)	12,144 ± 876
100	dUrd (100 μM)	8,308 ± 1,029
None	dAde (100 μM)	9,601 ± 1,079
100	dAde (100 μM)	5,180 ± 300
None	dGua (100 μM)	3,860 ± 170
100	dGua (100 μM)	2,522 ± 104
Experiment II		
None	None	9,605 ± 582
200	None	1,144 ± 71
None	dCyd (1 mM)	10,267 ± 492
200	dCyd (1 mM)	10,447 ± 105
None	dThd (1 mM)	1,199 ± 44
200	dThd (1 mM)	1,196 ± 136
None	dUrd (1 mM)	2,074 ± 111
200	dUrd (1 mM)	1,845 ± 58

* The cultures were performed in triplicate. The incorporation rate of ^{14}C-leucine was 500 cpm/10^5 cells/hr as explained in Materials and Methods.

metabolic nature of the compound, presumably acting as a thymidine analogue and requiring active cellular proliferation before its cytostatic effects.

5HmdUrd was readily catabolized to its corresponding base 5HmUra. It is noteworthy that 5HmUra is also a major catabolic product when dThd is administrated to patients with solid tumors: this observation made the authors question the role of 5HmUra in toxicity [24]. According to our present results, the toxicity of 5HmUra against HL-60 cells was negligible when compared to that of 5HmdUrd.

The phosphorolysis of 5-carbon substituted pyrimidine deoxyribonucleocides has been carefully studied: thymidine phosphorylase is highly specific for this class of compounds (see ref. 25). The relative rates of degradation of dThd and 5HmdUrd in the living cell system of this investigation paralleled differences in enzyme kinetics measured by Santi and his coworkers: relative V_{max}: dThd 1.0 and 5HmdUrd 0.60, and K_m: dThd 0.187 μM and 5HmdUrd 0.577 mM [25]. Hence, the similarity of the kinetics of the extracted enzyme [25] and the enzyme in the living cell system (this study) is emphasized. We showed that in the living cell system very little, if any, phosphorolysis of Urd takes place. Hence, it is highly conceivable that the phosphorolytic enzyme in living HL-60 cells is thymidine phosphorylase.

Human platelets are a rich source of enzymatic activity catabolizing 5-carbon-substituted pyrimidine deoxyribonucleosides and it has been observed previously that the rate of catabolism of 5HmdUrd by the platelet enzyme is slower than that of dThd [16, 17, 20]. This observation was confirmed in the present study. It is conceivable that platelets in human blood may be an important source of catabolizing activity if 5HmdUrd is given *in vivo*. We demonstrated here that catabolism by the platelet enzyme can be inhibited by 6-aminothymine, which is reported to be a specific inhibitor of thymidine phosphorylase [16, 17]. The significance of this information with regard to clinical chemotherapy remains to be evaluated.

The toxicity of 5HmdUrd against HL-60 cells was partially reversed by dThd. This, again, is consistent with the behavior of 5HmdUrd as a dThd antimetabolite. We were unable to demonstrate complete reversal of the action of 5HmdUrd by dThd, since the higher concentrations of dThd were toxic by themselves. On the other hand, complete reversal of the toxicity of 5HmdUrd was attained by using simultaneous high concentrations of dCyd. The biochemical mechanism of this antagonism is not fully understood at the molecular level. We would like to emphasize that in this respect 5HmdUrd is similar to dThd; dThd-induced inhibition of cell proliferation is proportional to the depletion of intracellular deoxycytidine triphosphate pools that result from blockade of ribonucleotide reductase [26, 27]. The toxicity of dThd is related to the endogenous intracellular dCTP pool as well as to the availability of dCyd that can be converted to dCTP via the pyridimidine salvage pathway [28]. Consistent with dCTP starvation in 5HmdUrd-treated cells is our finding that the incorporation of radioactive dCyd into DNA, dCDP and particularly into dCTP, via this salvage pathway was enhanced in a dose-dependent fashion when cells were exposed to 5HmdUrd.

5HmdUrd has many similarities to dThd as regards molecular structure and, as shown in the present investigation, as regards its metabolic behavior in culture. Nevertheless, the incoporation rate of the

Table 3. Effect of 5HmdUrd and dThd on the incorporation of radioactivity from [5-^{3}H]dCyd into DNA and into its precursor molecules during 15 min incubation *in vitro*

Treatment	Radioactivity incorporated (cpm/200,000 cells ± SD)*					
	DNA	dCTP	dCDP	dCMP	dCyd	Cyt
None	13,260 ± 230	2,850 ± 220	790 ± 26	1,040 ± 260	1,090 ± 310	900 ± 150
5HmdUrd (10^{-5} M)	23,420 ± 1,210	5,130 ± 410	1,120 ± 140	660 ± 130	610 ± 80	510 ± 75
5HmdUrd (10^{-4} M)	32,770 ± 1,890	13,880 ± 1,110	3,310 ± 290	800 ± 50	880 ± 290	1,160 ± 240
5HmdUrd (10^{-3} M)	32,020 ± 2,010	29,500 ± 2,410	4,540 ± 870	1,150 ± 200	770 ± 210	780 ± 55
dThd (10^{-3} M)	52,900 ± 2,480	43,580 ± 2,010	6,100 ± 1,410	1,460 ± 130	440 ± 30	620 ± 80

* The cultures were performed in triplicate. The counting efficiency for tritium was 27%.

compound into DNA is reported to be only 0.7–2.4% of that of dThd [11, 29] and it is conceivable that, unlike dThd, the compound may be excised from DNA by a specific glycosylase [30]. We propose that 5HmdUrd possesses peculiar characteristics and that the compound may be an interesting candidate for evaluation in clinical chemotherapy.

Acknowledgements—This investigation was supported by a grant from the Finnish Foundation for Cancer Research, and Grant No. Al 20480 awarded by the National Institute of Allergy and Infectious Disease of the National Institutes of Health, U.S.A.

REFERENCES

1. E. R. Kern, J. C. Overall and L. A. Glasgow, *J. infect. Dis.* **128**, 290 (1973).
2. K. K. Gauri (Ed.), *Adv. Ophthalmol.* **38**, 1 (1979).
3. E. De Clercq, J. Descamps, P. De Somer, P. J. Barr, A. S. Jones and R. T. Walker, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2947 (1979).
4. P. C. Maudgal, L. Missotten, E. De Clercq, J. Descamps and E. De Meuter, *Albrecht von Graefes Arch. Klin. Ophthalmol.* **216**, 261 (1981).
5. P. C. Maudgal, L. Dralands, L. Lamberts, E. De Clercq, J. Descamps and L. Missotten, *Bull. Soc. Belge Ophthal.* **193**, 49 (1981).
6. E. De Clercq, H. De Greef, J. Wildiers, G. De Jonge, A. Drochmans, J. Descamps and P. De Somer, *Br. Med. J.* **281**, 1178 (1980).
7. H. E. Kaufman and C. Heidelberger, *Science, N.Y.* **145**, 585 (1964).
8. C. Heidelberger, L. Griesbach, O . Cruz, R. j. Schnitzer and E. Grunberg, *Proc. Soc. exp. Biol. Med.* **97**, 470 (1958).
9. C. Heidelberger, in *Cancer Medicine* (Eds. J. F. Holland and E. Frei, III), p. 768. Lea & Febriger, Philadelphia (1973).
10. K. L. Mukherjee, J. Boohar, D. Wentland, F. J. Ansfield and C. Heidelberger, *Cancer Res.* **23**, 49 (1963).
11. L. I. Kahilainen, D. E. Bergstrom and J. A. Vilpo, *Acta chem. scand.* **B39**, 477 (1985).
12. C. K. Cain, M. F. Mallette and E. C. Taylor, Jr., *J. Am. chem. Soc.* **68**, 1996 (1946).
13. S. J. Collins, R. C. Gallo and R. E. Gallagher, *Nature, Lond.* **270**, 347 (1977).
14. T. R. Chen, *Exp. Cell Res.* **104**, 255 (1977).
15. B. B. Mishell, S. M. Shiigi, C. Henry, E. L. Chan, J. North, R. Gallily, M. Slomich, K. Miller J. Marbrook, D. Parks and A. H. Good, in *Selected Methods in Cellular Immunology* (Eds. B. B. Mishell and S. M. Shiigi), p. 3. W. H. Freedman, San Francisco (1980).
16. C. Desgranges, G. Razaka, M. Rabaud, P. Picard, F. Dupuch and H. Bricaud, *Biochem. Pharmac.* **31**, 2755 (1982).
17. C. Desgranges, G. Razaka, M. Rabaud, H. Bricaud, J. Balzarini and E. De Clercq, *Biochem. Pharmac.* **32**, 3583 (1983).
18. R. G. Stoller, C. E. Myers and B. A. Chabner, *Biochem. Pharmac.* **27**, 53 (1978).
19. A. F. Fleck and D. Begg, *Biochim. biophys. Acta* **108**, 333 (1965).
20. C. Desgranges, G. Razaka, M. Rabaud and H. Bricaud, *Biochim. biophys. Acta* **654**, 211 (1981).
21. M. Green, H. D. Barner and S. S. Cohen, *J. biol. Chem.* **228**, 621 (1957).
22. S. Waschke, J. Reefschläger, D. Bärwolff and P. Langen, *Nature, Lond.* **255**, 629 (1975).
23. E. De Clercq, J. Balzarini, P. F. Torrence, M. P. Mertes, C. L. Schmidt, D. Shugar, P. J. Barr, A. S. Jones, G. Verhelst and R. T. Walker, *Molec. Pharmac.* **19**, 321 (1981).
24. A. Leyva, J. H. Schornagel, J. Kraal, S. K. Wadman and H. M. Pinedo, *J. Cancer Res. Clin. Oncol.* **107**, 211 (1984).
25. C. Nakayama, Y. Wataya, R. B. Meyer, Jr. and D. V. Santi, *J. med. Chem.* **23**, 962 (1980).
26. N. Xeros, *Nature, Lond.* **194**, 682 (1962).
27. P. A. Morse and V. R. Potter, *Cancer Res.* **25**, 499 (1963).
28. A. N. Harris, E. C. Reynolds and L. R. Finck, *Cancer Res.* **39**, 538 (1979).
29. E. Matthes, D. Bärwolff, B. Preussel and P. Langen, *Antimetabol Biochem. Biol. Med. FEBS-Symp.* **57**, 115 (1979).
30. M. C. Hollstein, P. Brooks, S. Linn and B. N. Ames, *Proc. natn. Acad. Sci. U.S.A.* **81**, 4003 (1984).