Thymidylate synthetase-deficient mouse FM3A mammary carcinoma cell line as a tool for studying the thymidine salvage pathway and the incorporation of thymidine analogues into host cell DNA

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(Received 1 August 1983/Accepted 14 September 1983)

A thymidylate (dTTP) synthetase-deficient murine mammary carcinoma cell line (FM3A/TS⁻), auxotrophic for thymidine (dThd), proved extremely useful for studying the dependence of cell growth on the exogenous supply of dThd, the relation between cell growth and DNA synthesis, and the ability of a series of 25 5-substituted 2'-deoxyuridines (dUrd) to substitute for dThd in sustaining cell growth. FM3A/TS⁻ cells did not proliferate unless dThd was supplied to the cell culture medium. The 5-halogenated dUrd derivatives 5-chloro-dUrd, 5-bromo-dUrd and 5-iodo-dUrd also sustained FM3A/TS⁻ cell growth. The extents of incorporation of [*methyl*-³H]dThd and 5-iodo-[6-³H]dUrd into DNA were closely correlated with their stimulatory effects on FM3A/TS⁻ cell growth. This suggests that the stimulatory effects of the dUrd analogues on the growth rate of FM3A/TS⁻ cells may be considered as evidence for their incorporation into host cell DNA. Based on this premise it is postulated that, in addition to 5-chloro-dUrd, 5-bromo-dUrd, 5-iodo-dUrd and dThd itself, the following dThd analogues are also incorporated into FM3A/TS⁻ cell DNA (in order of the extent to which they are incorporated): 5-hydroxy-dUrd > 5-propynyloxy-dUrd > 5-ethyl-dUrd > 5-ethynyl-dUrd ~ 5-vinyl-dUrd. Thus, the dTMP synthetase-deficient FM3A/TS⁻ cell line represents a unique system to dissociate the *de novo* and salvage pathways of dTMP biosynthesis and to distinguish those dUrd analogues that are incorporated into DNA from those that are not.

The metabolic pathways that lead to dTTP are well established. dTTP may be produced by salvage of thymidine through the thymidine kinase reaction. This reaction is inhibited by dTTP, as shown in a number of different mammalian cell lines (Ives *et al.*, 1962; Maley & Maley, 1962a; Bresnick *et al.*, 1964; Hashimoto *et al.*, 1972). dTTP is also synthesized *de novo* by thymidylate synthetase through methylation of dUMP; dUMP may be produced by several pathways, all of which are inhibited by dTTP (Scarano *et al.*, 1960; Maley & Maley, 1962a,b, 1964; Moore & Hurlbert, 1966; Hashimoto *et al.*, 1972). Thus, dTTP appears to control its own synthesis by feedback inhibitory

effects, and as a consequence, dTMP synthetase is only indirectly regulated by these inhibitory effects, namely through a restriction in the availability of its substrate dUMP. Since it is the only enzyme involved in synthesis *de novo* of dTMP, dTMP synthetase can be regarded as an appropriate target in cancer chemotherapy. In addition to dTMP synthetase, dThd kinase is also an important enzyme not only for the utilization of exogenous dThd, but also for the activation of thymidine analogues such as 5-iodo-dUrd and 5-fluoro-dUrd which are currently used in virus and cancer chemotherapy (Heidelberger & Ansfield, 1963; Kaufman & Maloney, 1963). It is difficult to dissociate the *de novo* and salvage biosynthesis of thymidylate because of the complex regulatory interactions existing between both pathways.

Recently, a dTMP synthetase-deficient mutant cell line has been described which was derived

Abbreviations used: dThd, thymidine; dTTP, thymidylate; dTTP, thymidine 5'-triphosphate; dUrd, 2'-deoxyuridine; dUMP, 2'-deoxyuridine 5'-phosphate; dCyd, deoxycytidine; dCMP, 2'-deoxycytidine 5'-phosphate.

from mutagenized mouse FM3A carcinoma cells. This mutant cell line (FM3A/TS⁻) proved to be auxotrophic for thymidine (Ayusawa *et al.*, 1980, 1981; Koyama *et al.*, 1982). Thus, the dTMP synthetase-deficient cell line represents a unique system to dissect the *de novo* and salvage pathways of dTMP biosynthesis and can be used to distinguish those 5-substituted dUrd analogues that are incorporated into DNA from those that are not.

In this study, the activities *in vitro* and *in vivo* of dTMP synthetase and of the salvage enzymes dThd kinase and dUrd kinase in both FM3A/+ and FM3A/TS⁻ cells were assessed, and the dependence of FM3A/TS⁻ cell growth on the exogenous supply of dThd, the relation between cell growth and DNA synthesis and the ability of a series of 25 5-substituted 2'-deoxyuridines to substitute for dThd in sustaining FM3A/TS⁻ cell growth have further been established. The importance of this study is attested by the fact that compounds which are incorporated into cell DNA may be mutagenic, teratogenic or even carcinogenic. In this sense, the FM3A/TS⁻ cell line can be considered as a useful tool to detect and evaluate such compounds. Furthermore, FM3A/TS⁻ cells offer interesting perspectives for a detailed investigation of (i) the salvage pathway of dTMP biosynthesis, (ii) the labelling of host cell DNA with radioisotopes, and (iii) the biological consequences of incorporation of nucleoside analogues into host cell DNA.

Materials and methods

Cells

Mouse FM3A cells (subclone F28-7), originally established from a spontaneous mammary carcinoma in a C3H/He mouse (Ayusawa *et al.*, 1980), were grown in 75 cm² tissue culture flasks (Sterilin, Teddington, Middlesex, U.K.) in Eagle's minimal essential medium, supplemented with 10% (v/v) inactivated foetal calf serum (Gibco Bio-Cult, Glasgow, Scotland, U.K.), 2 mM-L-glutamine (Flow Laboratories, Irvine, Scotland, U.K.) and 0.075% (w/v) NaHCO₃ (Flow Laboratories). A FM3A subclone (designated as FM3A/TS⁻) was maintained in the same culture medium as described for the parent FM3A/+ cells, but this medium was supplemented with 20 µM-dThd.

Chemicals

The test compounds and their sources were as follows: dThd (Sigma), dUrd (Sigma), 5-fluoro-dUrd (Aldrich), 5-chloro-dUrd (Calbiochem-Behring), 5-bromo-dUrd (Sigma), 5-iodo-dUrd (Sigma), 5-trifluoromethyl-dUrd (P-L Biochemicals), 5-hydroxymethyl-dUrd (Calbiochem-Behring), 5-azidomethyl-dUrd (Kampf *et al.*, 1976; De

Clercq *et al.*, 1979b), 5-formyl-dUrd (Park *et al.*, 1980), 5-cyano-dUrd (Torrence *et al.*, 1977), 5-ethynyl-dUrd (Barr *et al.*, 1978; De Clercq *et al.*, 1979a), 5-vinyl-dUrd (De Clercq *et al.*, 1979a), (E)-5-(2-chlorovinyl)-dUrd (De Clercq *et al.*, 1979a), (E)-5-(2-bromovinyl)-dUrd (De Clercq *et al.*, 1979a; Jones *et al.*, 1979), (E)-5-(2-iodovinyl)-dUrd (De Clercq *et al.*, 1979a), 5-ethyl-dUrd (Swierkowski & Shugar, 1969; De Clercq & Shugar, 1975), 5-propyl-dUrd (De Clercq *et al.*, 1978), 5-methylthiomethyl-dUrd (De Clercq *et al.*, 1979b; Schmidt *et al.*, 1980), 5-methylsulphinylmethyl-dUrd (Schmidt *et al.*, 1980), 5-(1,3-dithiolan-2-yl)-dUrd (Park *et al.*, 1980), 5-thiocyano-dUrd (De Clercq *et al.*, 1975), 5-nitro-dUrd (provided by M. Robins, Edmonton, Alberta, Canada), 5-hydroxy-dUrd (Sefochem Fine Chemicals, Emek Hayarden, Israel), 5-propynyloxy-dUrd (Torrence *et al.*, 1978).

Radiochemicals

The radiolabelled nucleosides [*methyl*-³H]dThd (sp. radioactivity 47 Ci/mmol), [1',2'-³H]dUrd (sp. radioactivity 31 Ci/mmol), [5-³H]dCyd (sp. radioactivity 22 Ci/mmol) and [U-¹⁴C]dCyd (sp. radioactivity 462 mCi/mmol) were obtained from The Radiochemical Centre (Amersham, U.K.), and [5-³H]dUrd (sp. radioactivity 13 Ci/mmol) and 5-iodo-[6-³H]dUrd (sp. radioactivity 10 Ci/mmol) were obtained from Schwarz-Mann, (New York, NY, U.S.A.).

Examination of FM3A cell growth and incorporation of [*methyl*-³H]dThd, [1',2'-³H]dUrd and [5-³H]-dCyd into FM3A cell DNA

These methods have been described previously for mouse leukaemia L1210 cells (De Clercq *et al.*, 1981a).

dThd kinase, dUrd kinase and dTMP synthetase assay

The procedure for measuring cell-free dThd kinase, dUrd kinase and dTMP synthetase activities have been described previously (Balzarini *et al.*, 1982). The method for monitoring dUrd kinase activity was essentially the same as for dThd kinase. The substrate was dUrd instead of dThd. dTMP synthetase activity was measured in crude extracts of the FM3A cells instead of partially purified enzyme extracts as described by Balzarini *et al.* (1982).

³H release from [5-³H]dUrd and [5-³H]dCyd

dTMP synthetase activity in the intact FM3A cells was monitored by measuring ³H release from [5-³H]dUMP formed within the cells from [5-³H]dUrd or [5-³H]dCyd. ³H is released from [5-³H]dUMP during the conversion of [5-³H]dUMP

to dTMP by dTMP synthetase. Details of this procedure have been described by Balzarini & De Clercq (1984).

Results and discussion

Biochemical characterization of the FM3A/+ and FM3A/TS⁻ cell lines

Several procedures were followed to assess the activities *in vitro* and *in vivo* of dTMP synthetase and of the salvage enzymes dThd kinase and dUrd kinase in both FM3A/+ and FM3A/TS⁻ cells. The results are shown in Table 1.

There were no substantial differences between the activities *in vitro* of dThd kinase and dUrd kinase of FM3A/+ and FM3A/TS⁻ cells. However, dramatic differences were demonstrated for the dTMP synthetase activity. FM3A/TS⁻ cells showed less than 1% of the dTMP synthetase activity present in FM3A/+ cells (Table 1).

When measuring the incorporation *in vivo* of [*methyl*-³H]dThd and [1',2'-³H]dUrd into FM3A cell DNA, dThd was incorporated as effectively in FM3A/TS⁻ cell DNA as in FM3A/+ cell DNA, but dUrd was incorporated in FM3A/TS⁻ cell DNA at only 0.5% of that incorporated into the DNA of FM3A/+ cells. This is not surprising since the incorporation of dUrd into DNA occurs via the dTMP synthetase step. It is obvious, therefore, that cells lacking this enzyme cannot incorporate dUrd into their DNA.

We have also measured the incorporation of dCyd into cell DNA. dCyd can be incorporated into cell DNA as either dCMP or dTMP. The amount of dCyd that is incorporated as dCMP can be estimated by measuring the incorporation of [5-³H]dCyd into trichloroacetic acid-insoluble material. No [5-³H]dCyd can be detected as labelled dTMP in DNA, since the ³H at C-5 of the pyrimidine ring is exchanged for a methyl group in

the dTMP synthetase reaction. If [U-¹⁴C]dCyd is used as the radiolabelled precursor, incorporation of radioactive material into DNA occurs through both the dCyd→dCTP and the dCyd→dTTP pathways. By deducting the amount of radiolabelled material incorporated into DNA with [5-³H]dCyd as precursor from the amount of radiolabelled material incorporated into DNA with [U-¹⁴C]dCyd as the precursor, one can estimate how much dCyd is actually incorporated into DNA as dTMP (Balzarini & De Clercq, 1984). Since the dCyd→dTTP pathway passes through dTMP synthetase as the pivotal enzyme, the extent of incorporation of dCyd into DNA as dTMP reflects the cellular activity of dTMP synthetase (assuming that the dCMP deaminase activity is similar for both cell lines). In FM3A/+ cells, only 17.5% of the exogenous dCyd was incorporated into DNA as dCMP; the rest was incorporated as dTMP. Thus, in FM3A/+ cells the dCyd→dTTP pathway was six times more important than the dCyd→dCTP pathway. In FM3A/TS⁻ cells, however, the dCyd→dCTP pathway predominated, since more than 85% of the exogenous dCyd was incorporated into FM3A/TS⁻ cell DNA as dCMP (Table 1). These observations are compatible with the virtual absence of dTMP synthetase in FM3A/TS⁻ cells.

dTMP synthetase activity in intact cells can also be measured by direct evaluation of the ³H release from [5-³H]dUrd and [5-³H]dCyd (Balzarini & De Clercq, 1984). In FM3A/TS⁻ cells ³H release from [5-³H]dUrd and [5-³H]dCyd did not amount to more than 1–2% of the ³H release noted for FM3A/+ cells (Table 1). This again confirms the virtual absence of dTMP synthetase in FM3A/TS⁻ cells.

From the data presented in Table 1 it is clear that the FM3A/TS⁻ cell line is highly deficient in dTMP synthetase activity, while endowed with normal activity of the salvage enzyme dThd

Table 1. Biochemical characterization of the FM3A/+ and FM3A/TS⁻ cell lines
³H release was measured after 1 h incubation of the cells with the radiolabelled precursor.

	FM3A/+	FM3A/TS ⁻
Activity <i>in vitro</i> of:		
dTMP synthetase (pmol/h per mg of protein)	277	≤3
dThd kinase (nmol/h per mg of protein)	53.3	57.1
dUrd kinase (nmol/h per mg of protein)	100	119
Incorporation <i>in vivo</i> into cell DNA (pmol/24 h per 10 ⁵ cells) of:		
[<i>methyl</i> - ³ H]dThd	3.19	3.09
[1',2'- ³ H]dUrd	3.23	0.017
Ratio of incorporation of dCyd into cell DNA as dCMP to incorporation of dCyd into cell DNA as dTMP	0.212	6.40
³ H release (pmol equivalents of ³ H/h per 10 ⁷ cells) by intact cells from:		
[5- ³ H]dUrd (0.1 μM)	13.88	0.123
[5- ³ H]dCyd (0.1 μM)	7.3	0.149

Fig. 1. Growth curves for FM3A/+ (a) and FM3A/TS⁻ (b) cell lines upon addition of different concentrations of dThd

Concentrations of dThd: none (*), 0.605 µg/ml (×), 1.21 µg/ml (▽), 2.42 µg/ml (□), 4.84 µg/ml (△) and 9.68 µg/ml (○).

(dUrd) kinase. As a consequence, FM3A/TS⁻ cells are auxotrophic for dThd, since dTMP is no longer formed via the *de novo* pathway. As demonstrated in Fig. 1, FM3A/TS⁻ cells do not proliferate in the absence of exogenous dThd. However, in the presence of approx. 5–10 µg of exogenous dThd/ml, FM3A/TS⁻ cells grow equally well as FM3A/+ cells, and within the concentration range of 0.6–4.8 µg of exogenous dThd/ml, FM3A/TS⁻ cells grow up to a total number that is proportional to the dThd input (Fig. 1); within this concentration range doubling the concentration of dThd results in doubling the final cell number. Thus, the growth of the FM3A/TS⁻ cells shows a

Fig. 2. Relation between incorporation of [methyl-³H]dThd (a) or 5-iodo-[6-³H]dUrd (b) into DNA and cell growth at different concentrations of the compounds

stringent dependence on the exogenous supply of dThd, which makes the FM3A/TS⁻ cell line very useful for further studies of the dThd salvage pathway.

Relation between cell growth and DNA synthesis (incorporation of dThd into host cell DNA)

At suboptimal concentrations (<4.8 µg/ml) dThd did not alter the kinetics of FM3A/TS⁻ cell growth (i.e. generation time) but only reduced the final cell number. Apparently, cell proliferation was stopped as soon as all dThd had been consumed from the extracellular environment (Fig. 1b). At the optimal dThd concentration of 10 µg/ml no difference in cell growth could be observed between FM3A/TS⁻ and FM3A/+ cells (Fig. 1); at this dThd concentration, the initial cell number increased 7.6-fold during a 48 h incubation period, which is equivalent to about three cell generations with a generation time of 15 h. At supraoptimal concentrations (100–1000 µg/ml), dThd suppressed the growth of FM3A/TS⁻ cells (Figs. 2 and 3).

Inhibition of cell growth by high concentrations of dThd has been described previously (Bjursell & Reichard, 1973; Lowe & Grindey, 1976) and could

Fig. 3. Growth curves for FM3A/TS⁻ cells upon addition of different concentrations of dThd (a), 5-bromo-dUrd (b) and 5-iodo-dUrd (c)

Concentrations are: 1000 µg/ml (○), 100 µg/ml (△), 10 µg/ml (□) and 1 µg/ml (×).

be explained by an expansion of the dTTP pool, resulting in a decrease of the conversion of cytidine 5'-diphosphate to deoxycytidine 5'-diphosphate by the enzyme ribonucleotide diphosphate reductase. This would lead to a shortage of dCTP as substrate for DNA polymerase, a decrease in DNA synthesis, and, as a consequence, a delay in cell growth. Support for this explanation stems from the findings that addition of dCyd reverses the inhibitory effects of high dThd concentrations (Lowe & Grindey, 1976; Grant *et al.*, 1980). A similar observation was made for the inhibition of FM3A/TS⁻ cell growth at high dThd concentrations. Addition of 20–200 µg of dCyd/ml completely reversed the inhibitory effects of dThd (results not shown).

By using [*methyl*-³H]dThd and 5-iodo-[6-³H]dUrd, we could demonstrate that the pattern of FM3A/TS⁻ cell growth was closely correlated with host cell DNA synthesis, as monitored by the

incorporation of [*methyl*-³H]dThd and 5-iodo-[6-³H]dUrd, respectively. Over a broad range of concentrations varying from 0.1 to 10³ µg of [*methyl*-³H]dThd or 5-iodo-[6-³H]dUrd/ml, the dose-response curves for cell growth coincided with the dose-response curves for DNA synthesis, whether the latter was monitored by [*methyl*-³H]dThd (Fig. 2a) or 5-iodo-[6-³H]dUrd (Fig. 2b). Incorporation of dThd or 5-iodo-dUrd into DNA was most marked at a concentration of 10 µg/ml. This concentration gave also the highest increase in cell number. At dThd and 5-iodo-dUrd concentrations lower than 10 µg/ml, cell growth ceased after all compound was consumed and a proportionally smaller amount of nucleoside was incorporated into DNA (Fig. 2). At concentrations higher than 10 µg/ml, dThd and 5-iodo-dUrd inhibited FM3A/TS⁻ cell growth and at these concentrations the compounds were also less efficiently incorporated into DNA (Fig. 2). Thus, over a broad range of dThd and 5-iodo-dUrd concentrations cell growth and host cell DNA synthesis appeared to be closely correlated.

It is noteworthy that the inhibition of FM3A/TS⁻ cell growth by 5-iodo-dUrd or 5-bromo-dUrd was not reversed upon the addition of dCyd (results not shown). This suggests that the inhibitory effects of 5-iodo-dUrd and 5-bromo-dUrd on cell growth were achieved by a mechanism different from that of dThd. Goz (1978) reviewed the biological effects of 5-halogenated deoxyuridines in eucaryotic cell systems and concluded that almost all of the effects of 5-bromo-dUrd and 5-iodo-dUrd could be explained by their incorporation into DNA and that DNA containing 5-bromo-dUrd or 5-iodo-dUrd would not function normally.

When considering the growth curves of FM3A/TS⁻ cells cultured in the presence of different concentrations (1000, 100, 10 and 1 µg/ml) of dThd, 5-bromo-dUrd or 5-iodo-dUrd, four important observations could be made (Fig. 3).

(i) Irrespective of the nature and concentration of the nucleoside analogue, the FM3A/TS⁻ cells reached their stationary phase (final cell number) at the same time (approx. 60 h).

(ii) At optimal concentrations of the compounds (invariably 10 µg/ml for all three nucleosides), the cells attained the stationary phase after 2.4 generations for 5-bromo-dUrd, 1.8 generations for 5-iodo-dUrd, and 2.9 generations for dThd. The fewer generations noted for 5-bromo-dUrd and 5-iodo-dUrd may be related to a less efficient incorporation of the nucleoside analogues into DNA and/or lower replication rate of the 5-bromo-dUrd- or 5-iodo-dUrd-substituted DNA.

(iii) At lower concentrations of the compounds (1 µg/ml), the same growth kinetics were observed as with the optimal concentration until all sub-

Table 2. 5-Substituted dUrd derivatives as substitutes of dThd to sustain growth of FM3A/TS⁻ cells
An increase of cell number less than 5% of the initial inoculum could not be measured accurately and is therefore indicated by —.

Compound	Increase of cell number at optimal concentration of compound (% of inoculum)	Optimal concentration of compound ($\mu\text{g/ml}$)
dUrd	—	—
dThd	760	10
5-Fluoro-dUrd	—	—
5-Chloro-dUrd	528	10
5-Bromo-dUrd	552	10
5-Iodo-dUrd	344	10
5-Trifluoromethyl-dUrd	—	—
5-Hydroxymethyl-dUrd	—	—
5-Azidomethyl-dUrd	—	—
5-Formyl-dUrd	—	—
5-Cyano-dUrd	—	—
5-Ethynyl-dUrd	160	10
5-Vinyl-dUrd	152	10
(E)-5-(2-Chlorovinyl)-dUrd	—	—
(E)-5-(2-Bromovinyl)-dUrd	—	—
(E)-5-(2-Iodovinyl)-dUrd	—	—
5-Ethyl-dUrd	272	300
5-Propyl-dUrd	—	—
5-Methylthiomethyl-dUrd	—	—
5-Methylsulphinylmethyl-dUrd	—	—
5-(1,3-Dithiolan-2-yl)-dUrd	—	—
5-Thiocyano-dUrd	—	—
5-Nitro-dUrd	—	—
5-Hydroxy-dUrd	512	300
5-Propynyloxy-dUrd	304	1000

strate was consumed. Then, an immediate and complete stop of cell growth ensued.

(iv) At higher concentrations of compounds (100 or 1000 $\mu\text{g/ml}$), the cells grew at a considerably lower rate. This may be due to a direct inhibition of DNA replication or shut off in the supply of precursors for DNA synthesis, resulting in retardation of the synthesis of DNA and the subsequent growth rate.

Ability of 5-substituted 2'-deoxyuridines to substitute for dThd in sustaining FM3A/TS⁻ cell growth

A series of 25 5-substituted dUrd derivatives were evaluated at concentrations ranging from 0.1 to 1000 $\mu\text{g/ml}$ for their effects on FM3A/TS⁻ cell growth. Any cell growth that could be observed following replacement of exogenous dThd by the dThd analogue may be considered as evidence for the incorporation of the dThd analogue into host cell DNA. There were marked differences in the ability of the dThd analogues to substitute for dThd in sustaining FM3A/TS⁻ cell growth (Table 2). Most compounds, i.e. 5-fluoro-dUrd, 5-hydroxymethyl-dUrd, 5-azidomethyl-dUrd, (E)-5-(2-bromovinyl)-dUrd, 5-propyl-dUrd, and several others,

failed to stimulate cell growth of the FM3A/TS⁻ cells. Only nine dThd analogues were able to stimulate cell growth. These are the compounds presented in Fig. 4.

5-Chloro-dUrd and 5-bromo-dUrd emerged as the most efficient alternative substrates for cell growth (Fig. 4). The highest yield was observed if the compounds were added at a concentration of 3–30 $\mu\text{g/ml}$. Within this concentration range the cell number was increased 5.0–5.3-fold by 5-chloro-dUrd, and 4.6–5.5-fold by 5-bromo-dUrd. This is slightly lower than the 6.1–7.6-fold increase in cell number effected by dThd. For 5-iodo-dUrd, the increase in cell number was only 3.4-fold. 5-Vinyl-dUrd and 5-ethynyl-dUrd were also capable of stimulating FM3A/TS⁻ cell growth, but the increment in cell number that they achieved was relatively meagre (less than 2-fold). Furthermore, 5-ethyl-dUrd, 5-propynyloxy-dUrd and 5-hydroxy-dUrd effected a substantial increase in cell number (2.7-, 3.0- and 5.1-fold, respectively), but only at the higher concentration range (300–1000 $\mu\text{g/ml}$). Finally, 5-methyl-dCyd brought about a 6.4-fold increase in cell population when added to the cells at a concentration of 300 $\mu\text{g/ml}$. That 5-methyl-

Fig. 4. 5-Substituted dUrd and dCyd derivatives as substitutes of dThd to sustain growth of FM3A/TS⁻ cells. The initial cell inoculum at time zero was 250 000 cells/ml. Cells were incubated for 48 h in the presence of the compounds at the indicated concentrations.

dCyd would be a good alternative substrate for cell growth is not surprising, since the compound may be readily deaminated to the corresponding dThd analogue, either at the nucleoside level (by dCyd deaminase) or at the nucleotide level (by dCMP deaminase).

In a previous study (De Clercq *et al.*, 1981b), we examined the effects of 5-substituted dUrd analogues for their ability to induce retrovirus particles in mouse Balb/3T3 cells. As a prerequisite for the induction of retrovirus particles from carrier cell lines the compound should be incorporated into the host cell DNA (Teich *et al.*, 1973). A number of compounds, i.e. 5-iodo-dUrd, 5-bromo-dUrd and 5-propynyloxy-dUrd, which were found to substitute for dThd in sustaining FM3A/TS⁻

cell growth and are therefore postulated to be incorporated into host cell DNA, have also been shown to trigger the release of retrovirus particles (De Clercq *et al.*, 1981b). Other compounds, i.e. 5-ethyl-dUrd and 5-vinyl-dUrd, which are known to be incorporated into cellular DNA (Chelton *et al.*, 1973; Swierkowska *et al.*, 1973) and, accordingly, substituted for dThd in sustaining FM3A/TS⁻ cell growth, failed to induce the production of oncornavirus particles at non-toxic concentrations (De Clercq *et al.*, 1981b). So did 5-ethynyl-dUrd, although our present data indicate that to a small extent it may be incorporated into host cell DNA. Most dThd analogues, however, did not substitute for dThd in stimulating FM3A/TS⁻ cell growth and, likewise, failed to trigger the release of

oncornavirus particles. Foremost among the latter compounds are the 5-(2-halogenovinyl)-substituted dUrd analogues, i.e. (*E*)-5-(2-bromovinyl)-dUrd. The observation that they did not stimulate the growth of FM3A/TS⁻ cells can be considered as indirect evidence that they are not incorporated into host cell DNA.

In view of the potentials of dUrd analogues, i.e. 5-fluoro-dUrd, 5-iodo-dUrd, 5-trifluoromethyl-dUrd and (*E*)-5-(2-bromovinyl)-dUrd, as either anticancer or antiviral agents, it is extremely important to establish whether these compounds can be incorporated into normal host cell DNA. If incorporated, these compounds may be mutagenic, teratogenic or even carcinogenic. The dTMP synthetase-deficient cell line FM3A/TS⁻, which is auxotrophic for dThd, appears to be a useful test system to assess whether dThd analogues are incorporated into cellular DNA. It is inferred that those dThd analogues that, akin to dThd itself, stimulate the growth of FM3A/TS⁻ cells, can only do so because they are incorporated into the host cell DNA.

This investigation was supported by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Krediet no. 30048.75) and the Geconcentreerde Onderzoeksacties (Conventie no. 81/86-27). We thank Miette Stuyck and Lizette Van Berckelaer for excellent technical help and Christiane Callebaut for editorial assistance.

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