Thymidylate synthetase-positive and -negative murine mammary FM3A carcinoma cells as a useful system for detecting thymidylate synthetase inhibitors

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The murine mammary FM3A/O and the thymidylate (dTMP) synthetase-deficient FM3A/TS$^-$ carcinoma cell lines can be considered as a novel and useful test system for the detection of nucleoside analogues which are directly aimed at the thymidylate synthetase. These compounds should be inhibitory for FM3A/O but not for FM3A/TS$^-$ cells, and their inhibitory effects on FM3A/O cell growth should be readily reversed by exogenous dThd within the concentration range of 5–20 μM.

Murine mammary FM3A carcinoma *Thymidylate synthetase* *5-Substituted 2'-deoxyuridine analog*

1. INTRODUCTION

A broad variety of nucleoside analogues have been developed as potential antitumor agents, and the biochemical basis of their cytostatic action has been extensively studied using murine leukemia L1210 cells as a test system. 5-Substituted 2'-deoxyuridines such as 5-fluoro-dUrd [1–4], 5-trifluoromethyl-dUrd [1,5,6], 5-nitro-dUrd [1,7,8] and 5-formyl-dUrd [1,9,10] suppress tumor cell proliferation by inhibition of thymidylate (dTMP) synthetase. This enzyme is pivotal for the de novo biosynthesis of DNA and can, therefore, be regarded as an attractive target for cancer chemotherapy. Not surprisingly, its activity is substantially elevated in proliferating cells, as compared to normal tissues [11]. The interaction of pyrimidine nucleoside analogues with thymidylate synthetase has been investigated either directly with isolated or purified enzyme preparations [12–17] or indirectly in intact cells, by measuring (i) the differential incorporation of radiolabelled dUrd and dThd into DNA [1], (ii) the reversal of the cell growth inhibition by dUrd and dThd [1], and (iii) tritium release from [5-^{3}H]dUrd or [5-^{3}H]dCyd [18–21].

We propose here a novel system for the evaluation and detection of dTMP synthetase inhibitors. This system is based upon the use of a murine FM3A mammary carcinoma FM3A/O cell line and its dTMP synthetase-deficient subline FM3A/TS$^-$. For a series of twenty 5-substituted 2'-deoxyuridines there was a close correlation between their inhibitory effects on FM3A/O cell growth and their inhibitory effects on L1210 cell growth (r = 0.986). For those compounds that exerted their cytostatic activity by an inhibitory effect at the dTMP synthetase level, viz., 5-fluoro-dUrd, 5-trifluoromethyl-dUrd, 5-nitro-dUrd, 5-ethynyl-dUrd and 5-formyl-dUrd, addition of exogenous dThd brought about a pronounced reversal of the FM3A/O cell growth-inhibiting effects. This reversal was dose-dependent within the dThd concentration range 5–20 μM. The FM3A/TS$^-$ cells did not grow in the absence of dThd, and when assayed in the presence of 5 or 20 μM dThd, none of the aforementioned 5-substituted 2'-deoxyuridines exerted a marked inhibitory effect on the growth of

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FM3A/TS$^-$ cells. This observation corroborates the hypothesis that the cytostatic action of 5-substituted 2'-deoxyuridines such as 5-fluoro-dUrd, 5-trifluoromethyl-dUrd, etc. is primarily targeted at the dTMP synthetase level.

2. MATERIALS AND METHODS

2.1. *Cells*

Mouse leukemia L1210 cells were grown in 75-cm^2 tissue culture flasks (Sterilin, Teddington, England) in Eagle's minimal essential medium, supplemented with 10% (v/v) inactivated foetal calf serum (Gibco Bio-Cult, Glasgow, Scotland), 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland) and 0.075% (w/v) $NaHCO_3$ (Flow Laboratories).

Mouse FM3A cells (subclone F28-7), originally established from a spontaneous mammary carcinoma in a C3H/He mouse [22,23] and designated FM3A/O, were grown in the same culture medium as the L1210 cells. A FM3A subclone (designated FM3A/TS$^-$) was also maintained in this culture medium, but supplemented with 20 μM dThd [24].

2.2. *Chemicals*

The sources of the compounds were as follows: 5-fluoro-dUrd (Aldrich, Milwaukee, WI); 5-trifluoromethyl-dUrd (P.-L. Biochemicals, Milwaukee, WI); 5-nitro-dUrd (provided by M.J. Robins, Edmonton, Canada); 5-ethynyl-dUrd [25,26], 5-formyl-dUrd [27], 5-hydroxymethyl-dUrd (Calbiochem-Behring, Lucerne), 5-vinyl-dUrd [26], (*E*)-5-(2-iodovinyl)-dUrd [26], (*E*)-5-(2-bromovinyl)-dUrd [26,28], 5-ethyl-dUrd [29,30], 5-azido-2methyl-dUrd [31,32], 5-cyano-dUrd [33], (*E*)-5-(2-chlorovinyl)-dUrd [26], 5-methylthiomethyl-dUrd [32,34], 5-(1,3-dithiolan-2-yl)-dUrd [27], 5-propyl-dUrd [35], 5-methylsulfinylmethyl-dUrd [34], 5-hydroxy-dUrd (Sefochem, Emek Hayarden, Israel), 5-propynyloxy-dUrd [36], dUrd (Sigma, MO), dThd (Sigma).

2.3. *Inhibition of tumor cell growth*

All assays were performed in microtest plates (Sterilin). To each well were added 5×10^4 L1210, FM3A/O or FM3A/TS$^-$ cells and a given amount of the test compound. In those assays that were aimed at evaluating the effects of dThd, 5 or 20 μM dThd was added to the cells together with varying amounts of the test compound. The doses used for dThd (5 and 20 μM) corresponded to their capacity to sustain normal growth of the FM3A/TS$^-$ cells during 24 and 48 h, respectively. The cells were allowed to proliferate for 24 and 48 h at 37°C in a humidified, CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter Counter (Coulter Electronics, Harpenden, England).

3. RESULTS AND DISCUSSION

3.1. *Comparison of the FM3A/O and L1210 cell systems for the evaluation of the inhibitory effects of 5-substituted 2'-deoxyuridines on cell proliferation*

The 5-substituted 2'-deoxyuridines were examined at a variety of concentrations (1 ng/ml to 1 mg/ml) for their inhibitory effects on FM3A/O cell proliferation, and the ID_{50} values (inhibitory dose-50, or dose required to inhibit cell proliferation by 50%) are presented in table 1. There were marked differences in the inhibitory effects of the 5-substituted 2'-deoxyuridines on FM3A/O cell growth. With an ID_{50} of 1 ng/ml, 5-fluoro-dUrd was the most potent, and with an ID_{50} of >1 mg/ml, 5-propyl-, 5-propynyloxy-, 5-hydroxy- and 5-methylsulfinylmethyl-dUrd were the least potent agents. The other compounds showed ID_{50} values that were intermediate between these two extremes. Clearly, when compared with the ID_{50} values previously found for L1210 cell proliferation [1,37], the most potent inhibitors of FM3A/O cell growth (with an $ID_{50} < 1$ μg/ml), viz., 5-fluoro-dUrd, 5-trifluoromethyl-dUrd, 5-nitro-dUrd, 5-ethynyl-dUrd and 5-formyl-dUrd, were also the most inhibitory to L1210 cell growth (fig.1); and those compounds that were inactive as inhibitors of FM3A/O cell growth were also inactive against L1210 cells. In general, the ID_{50} values for the mouse mammary FM3A/O carcinoma cells corresponded closely to the ID_{50} values for L1210 cells, except for (*E*)-5-(2-iodovinyl)-dUrd and 5-cyano-dUrd, which were about 5–7-times more inhibitory to FM3A/O cells. For the 20 dUrd analogues that were evaluated for inhibition of tumor cell growth there appeared to be a strong

Table 1

Inhibitory effects of 5-substituted 2'-deoxyuridine derivatives on the proliferation of FM3A/O cells

No.	Compound	ID_{50}[a] (μg/ml) for FM3A/O cell proliferation		
		As such	Upon addition of 5 μM dThd	Upon addition of 20 μM dThd
1	5-fluoro-dUrd	0.001 (±0.0003)	0.030 (±0.02)	124 (±56)
2	5-trifluoromethyl-dUrd	0.013 (±0.007)	0.088 (±0.013)	180 (±30)
3	5-nitro-dUrd	0.013 (±0.003)	0.045 (±0.022)	>1000
4	5-ethynyl-dUrd	0.131 (±0.005)	0.790 (±0.010)	152 (±96)
5	5-formyl-dUrd	0.171 (±0.062)	1.5 (±0.54)	150 (±78)
6	5-hydroxymethyl-dUrd	2.0 (±0.3)	55 (±4.6)	351 (±45)
7	5-vinyl-dUrd	2.0 (±0.65)	28 (±14.3)	252 (±118)
8	(*E*)-5-(2-iodovinyl)-dUrd	4.0 (±0.91)	201 (±54)	315 (±31)
9	(*E*)-5-(2-bromovinyl)-dUrd	11 (±5.07)	380 (±227)	529 (±197)
10	5-ethyl-dUrd	12 (±0.02)	554 (±136)	>1000
11	5-azidomethyl-dUrd	26 (±2.0)	64 (±13)	62 (±18)
12	5-cyano-dUrd	30 (±4.61)	268 (±17)	>1000
13	(*E*)-5-(2-chlorovinyl)-dUrd	184 (±27)	290 (±39)	573 (±97)
14	5-methylthiomethyl-dUrd	420 (±50)	349 (±86)	507 (±36)
15	5-(1,3-dithiolan-2-yl)-dUrd	537 (±153)	587 (±47)	>1000
16	5-propyl-dUrd	>1000	>1000	>1000
17	5-methylsulfinylmethyl-dUrd	>1000	>1000	>1000
18	5-hydroxy-dUrd	>1000	>1000	>1000
19	5-propynyloxy-dUrd	>1000	>1000	>1000
20	dUrd	>1000	>1000	>1000

[a] Inhibitory dose-50, ± SD

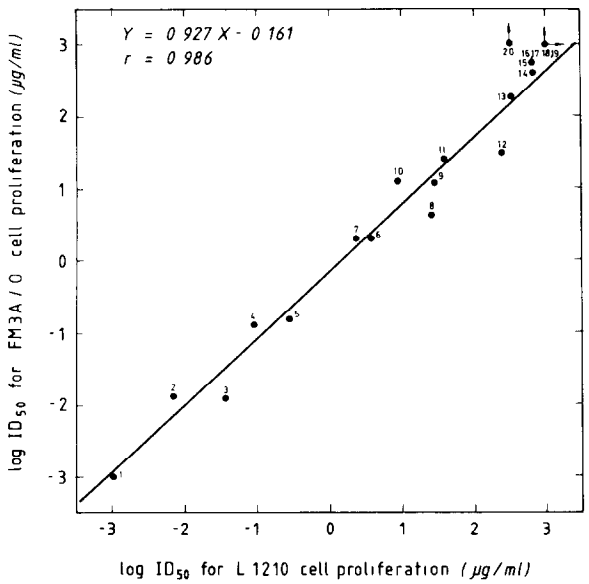

Fig.1. Correlation between the log ID_{50} of 5-substituted 2'-deoxyuridines for L1210 cell proliferation and their log ID_{50} for FM3A/O cell proliferation. The ID_{50} values for L1210 cell proliferation are taken from [1]. The symbols for the compounds are indicated in table 1.

linear correlation (r = 0.986) between the log ID_{50} for FM3A/O cell growth and that for L1210 cell growth (fig.1). Thus, from the results obtained with a given 5-substituted 2'-deoxyuridine in the mouse mammary FM3A/O carcinoma system, one might readily predict its inhibitory activity for murine leukemia L1210 cells and vice versa. Also, the L1210 and FM3A/O cell lines showed similar ID_{50} values for other antitumor agents such as cytosine arabinoside, 5-fluorouracil and methotrexate (not shown). Therefore, FM3A/O cells may be considered as a valuable system for the evaluation of compounds with potential antitumor activity. Moreover, when 10^6 FM3A/O cells were injected intraperitoneally into C_3H/He mice, the median life span of the mice was about 17 days, as compared to 6 days for DBA/2 mice inoculated with murine leukemia L1210 cells and 11 days for DBA/2 mice inoculated with murine leukemia P388 cells. Thus, the murine mammary (FM3A) carcinoma model can also be advocated for the in vivo evaluation of antitumor agents.

3.2. *Inhibitory effects of 5-substituted 2'-deoxyuridines on the proliferation of FM3A/O and FM3A/TS⁻ cells in the presence of thymidine*

Recently, a dTMP synthetase-deficient mutant cell line was derived from mutagenised mouse FM3A carcinoma cells [22,23]. This mutant cell line resulted from a genetic defect in dTMP synthetase and proved to be very stable. Less than 1% of dTMP synthetase activity was detected in the mutant FM3A/TS⁻ cells, as compared to the parent wild-type FM3A/O cells [24]. However, there was no substantial difference between the dUrd kinase and dThd kinase activities of FM3A/O and FM3A/TS⁻ cells [24]. Since the FM3A/TS⁻ cell line was auxotrophic for dThd [24,38], it did not grow unless exogenous dThd was supplied [24]. For normal cell growth 20 μM dThd was required; 5 μM dThd only enabled the cells to proliferate for 24 h, whereafter cell growth reached the stationary growth phase [24]. This means that to evaluate the inhibitory effects of 5-substituted 2'-deoxyuridines on FM3A/TS⁻ cell growth, dThd had to be added to the cell culture medium. Two different concentrations (5 and 20 μM) of dThd were used, and both FM3A/O and FM3A/TS⁻ cells were assayed for the sensitivity to the growth-inhibitory effects of 5-substituted 2'-deoxyuridines in the presence of these dThd concentrations.

For those compounds, which are assumed to interact at the dTMP synthetase level, viz. compounds 1–5, there was a marked difference between their ID_{50} values for FM3A/O cell growth upon addition of 5 μM dThd and those upon addition of 20 μM dThd (table 1). The ratio between these ID_{50} values was as great as 2000 for 5-trifluoromethyl-dUrd, 4000 for 5-fluoro-dUrd and >20000 for 5-nitro-dUrd. No such differences

Table 2

Inhibitory effects of 5-substituted 2'-deoxyuridine derivatives on the proliferation of FM3A/TS⁻ cells

No.	Compound	ID_{50}[a] (μg/ml) for FM3A/TS⁻ cell proliferation	
		Upon addition of 5 μM dThd	Upon addition of 20 μM dThd
1	5-fluoro-dUrd	159 (±91)	91 (±5.5)
2	5-trifluoromethyl-dUrd	25 (±15)	167 (±53)
3	5-nitro-dUrd	>1000	>1000
4	5-ethynyl-dUrd	>100	>100
5	5-formyl-dUrd	270 (±33)	270 (±5)
6	5-hydroxymethyl-dUrd	60 (±25)	316 (±35)
7	5-vinyl-dUrd	203 (±168)	271 (±40)
8	(*E*)-5-(2-iodovinyl)-dUrd	255 (±72)	258 (±34)
9	(*E*)-5-(2-bromovinyl)-dUrd	297 (±7.9)	373 (±110)
10	5-ethyl-dUrd	734 (±19)	>1000
11	5-azidomethyl-dUrd	171 (±90)	40 (±7)
12	5-cyano-dUrd	>1000	>1000
13	(*E*)-5-(2-chlorovinyl)-dUrd	226 (±41)	507 (±85)
14	5-methylthiomethyl-dUrd	269 (±86)	398 (±31)
15	5-(1,3-dithiolan-2-yl)-dUrd	375 (±193)	648 (±140)
16	5-propyl-dUrd	427 (±46)	>1000
17	5-methylsulfinylmethyl-dUrd	>1000	>1000
18	5-hydroxy-dUrd	>1000	>1000
19	5-propynyloxy-dUrd	>1000	>1000
20	dUrd	>1000	>1000

[a] Inhibitory dose-50; ± SD

The ID_{50} values of the compounds as such (in the absence of dThd) could not be determined since the FM3A/TS⁻ cells did not proliferate in the absence of dThd

were found in the ID_{50} values for FM3A/TS$^-$ upon addition of 5 μM dThd and 20 μM dThd, respectively (table 2). In fact, none of the 5-substituted 2′-deoxyuridines exerted a pronounced inhibitory effect on FM3A/TS$^-$ cell growth, irrespective of the dThd concentration used. The fact that compounds 1–5, while strongly inhibitory to FM3A/O cells, were virtually inactive against FM3A/TS$^-$ cells, combined with the observation that their inhibitory effect on FM3A/O cell growth was reversed by dThd in a dose-dependent fashion, clearly indicates that these compounds are targeted at the dTMP synthetase level.

The cytostatic effects of compounds 6–10 were also reversed upon the addition of 5 μM dThd (14–50-fold); but only a slight increment in the reversal ratio was observed by further increasing the dThd concentration to 20 μM. As noted in [1], addition of dUrd considerably reversed the cytostatic effects of compounds 6–10, whereas addition of dThd caused only a slight further decrease in the inhibitory effects of these compounds on cell proliferation. For 5-substituted 2′-deoxyuridines to qualify as inhibitors of dTMP synthetase, addition of dThd should give a significantly greater reversal of the tumor growth-inhibiting effects than addition of dUrd [1]. This is clearly the case for compounds 1–5 but not for compounds 6–10. Therefore, the latter cannot be considered as truly specific or potent inhibitors of dTMP synthetase, and this conclusion is further borne out by the results obtained here.

As a rule, none of the 5-substituted 2′-deoxyuridines proved to be more inhibitory toward FM3A/TS$^-$ than FM3A/O cells. This is evident from a direct comparison of the ID_{50} values of the compounds for FM3A/TS$^-$ and FM3A/O cells in the presence of 5 and 20 μM dThd, respectively (tables 1,2).

In conclusion, the murine mammary FM3A carcinoma (FM3A/O-FM3A/TS$^-$) cell lines can be considered as a novel and useful test system for the detection of thymidylate synthetase inhibiting nucleoside analogues.

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