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Selective antiproliferative effects of thymidine

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Abstract. A substance with antiproliferative bioactivity in an aqueous extract of *Cordyline terminalis* was purified and identified by mass spectrometry to be the natural nucleoside, thymidine. 10^{-5} M Thymidine inhibited EL4 cell replication and decreased cell viability after 12–24 h. The effect was highly specific for this nucleoside. Treated cell cultures showed a significant increase in S phase cells and a corresponding decrease in G_1 phase cells. Nitrobenzylthioinosine (which prevented facilitated entry of thymidine) protected cells from the antiproliferative action of thymidine. A human breast cancer cell line (MCF7) was also growth-inhibited by 10^{-5} M thymidine but a murine lymphoma cell line (K36) was not. Thus, submillimolar thymidine has effects on cell proliferation which are selective both with respect to specificity for the compound and for different tumour cell lines.
Key words. Thymidine; nucleoside; lymphoma; cell cycle; cell proliferation.

A significant number of drugs owe their existence to the discovery of biological activities in natural products, especially those of plant origin. Among several reasons for seeking to preserve the biodiversity of tropical rain forests is a general recognition that these areas are a potentially rich repository of yet undiscovered pharmaceutical agents. In the course of a screening program aimed at identifying antitumour compounds in tropical plants, we prepared aqueous extracts from the leaves of *Cordyline terminalis* (L.) *Kunth* (*Agavaceae*). Although this plant has a tradition of medicinal use, none of its recorded uses includes treatment of tumours[1].

We now report that antiproliferative bioactivity present in aqueous extracts of *Cordyline terminalis* is due to thymidine. This natural nucleoside selectively inhibits growth of lymphoma and breast cancer cells – a property not shared by thymine or by other nucleosides.

Materials and methods

Cell cultures. Two murine lymphoma cell lines (EL4 and K36) and a human breast cancer-derived cell line (MCF7) were propagated as previously described[2,3].

Assays for antiproliferative activity. Three methods were used: 1) enumeration of cell density and viable cells by trypan blue exclusion using a Neubauer haemocytometer, 2) quantitation of acid-resistant macromolecular [^{3}H]thymidine incorporation[3], and 3) a colorimetric method using a tetrazolium salt (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide, MTT). Conversion of MTT to a coloured formazan compound by intact mitochondria is a measure of cell number and viability[4]. The amount of formazan compound formed was quantitated by measuring absorbance at 570 nm. The purification scheme was monitored using EL4 cells dispensed in 0.18 ml aliquots (500,000 cells/ml) into wells of a sterile 96-well plate. Duplicate wells received 0.02 ml of the aqeous extract (or chromatographic fractions thereof). Control wells received an identical volume of solvent alone. After 3 h, cells were pulsed for 1 h with 37 kBq [^{3}H]thymidine/well (248 GBq/mmol, Amersham) following which they were trapped on scintillant-coated glass fiber filters (Beckman Ready Filters) using a Brandel cell harvester and washing steps as previously described[2]. Data are expressed by setting [^{3}H]thymidine incorporation obtained in the presence of solvent alone at 100%.

Aqueous extraction. Cordyline terminalis leaves were oven-dried (50 °C) and milled. Fifty-nine g of milled powder was boiled in 650 ml water for 1 h, filtered and lyophilized to dryness. The lyophilized residue (2.1 g) was dissolved in 35 ml water. A clear saturated solution (obtained by centrifugation for 10 min at 20,000 g) was the starting material for isolation of antiproliferative bioactivity.

Sequence of chromatographic separations. All chromatographic columns were equilibrated with water. Although crude aqueous plant extracts were initially screened for their effects on cell density and viability as well as for effects on [^{3}H]thymidine incorporation, UV (254 nm)-absorbing fractions from each purification step were assayed for inhibition of [^{3}H]thymidine incorporation. Only fractions which inhibited [^{3}H]thymidine incorporation in EL4 cells were carried forward to the next purification step. Aqueous extract (32 ml) was applied to Sephadex G25 (Pharmacia) column (2.6 × 66 cm) from which 36 fractions of 35 ml each were collected. Fraction numbers 10–12 were pooled, lyophilized and reconstituted in water to saturation. The active pool was next passed over a Sephadex G10 (Pharmacia) column (2.6 × 66 cm). Fraction numbers 7–10 (35 ml

each) from this column were pooled, concentrated to 3.6 ml and applied to a column of quaternary amine silane-bonded to silica gel (J. T. Baker) (1.6 × 36 cm). Bioactive fractions in the column flow-through were pooled and concentrated 7.3-fold prior to fractionation on a carboxymethyl cellulose (Whatman CM23) cation exchange column (2.6 × 30 cm). Forty fractions (8 ml each) were collected in the flow-through. Of these, five fractions (numbers 19–23) were pooled, concentrated 5-fold and applied to a Sephadex G25 column (2.6 × 35 cm). The single fraction (4.65 ml) from this step which had the highest activity was finally purified by high-performance liquid chromatography (HPLC) on a C18 column (4.6 × 250 mm). Sample size was 0.1 ml and flow rate 1 ml/min. After a 2-minute water wash, the column was eluted with a water-methanol gradient (0%–100% methanol). Four UV-absorbing peaks were obtained; the peak with highest bioactivity emerged last (12.96 min., 35% methanol). This fraction was manually collected and dried in a centrifugal evaporator.

Mass spectrometry. The mass spectrum of the most highly purified fraction from HPLC was determined on a VG 7035 micromass mass spectrometer at a source temperature of 200 °C with an ion current of 70 eV.

Cell cycle analysis. This was performed on a FACScan cytometer (Becton Dickinson, USA) equipped with an air-cooled 488 nm, 15 mW argon laser. One million cells were suspended in 1 ml phosphate-buffered saline (0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4 and 0.002 M KH_2PO_4, pH 7.4) containing 7.5×10^{-5} M propidium iodide. Chicken erythrocytes were added to samples as an internal standard. One ml of modified Vindelov's solution (0.01 M Tris, pH 8/1 mM NaCl/ 7.5×10^{-5} M propodium iodide containing RNase A 0.7 g/L, Nonidet P-40 1 ml/L) was added and the mixture vortexed for 15 s. Cells were left to stain for 30–60 min at room temperature. Distribution of cells among the cell cycle phases was obtained by analysis of DNA histogram data using Cell Fit software.

Results

Antigrowth activity present in the aqueous leaf extract of *Cordyline terminalis* was identified initially by screening for effects on cell density, cell viability and [^{3}H]thymidine incorporation. Results of the purification scheme are shown in sequence in figures 1 (A–E) and 2. The most highly purified bioactive fraction obtained at the final step (HPLC) was a UV-absorbing peak eluting at 12.96 min which was analyzed by direct probe mass spectrometry (fig. 3). This showed a molecular ion at m/z 242 (3.5%) corresponding to the molecular mass of thymidine. It showed fragmentation peaks typical of nucleosides, in which the main cleavage occurs between the sugar (S) and the base (B) residue. Thus the base peak of the 2-deoxysugar (S) occurs at m/z 117 (100%) and typical fragmentation peaks corresponding to

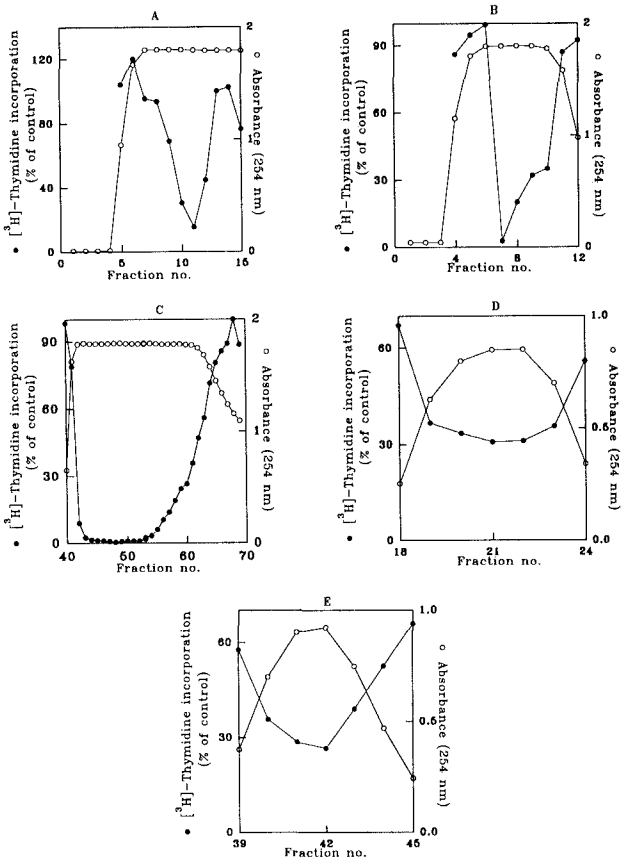

Figure 1. Sequential fractionation scheme in purification of bioactivity. *A* Sephadex G25, *B* Sephadex G10, *C* Quaternary amine, *D* Carboxymethyl cellulose, *E* Sephadex G25. Open circles denote absorbance at 254 nm and filled circles denote [^{3}H]thymidine incorporation as percentage of the control value (water alone).

(B + 1), (B + 2), (B + 30) and (M-89) are found at m/z 126 (43%), 127 (32%), 155 (0.5%) and 153 (4%), respectively. The (B + 1) fragment is stronger than the (B + 2) fragment – a characteristic feature of 2-deoxysugars[5]. The identity of the sample was further substantiated by direct comparison with the mass spectrum of an authentic specimen of thymidine determined under identical conditions.

Thus the strategy adopted for purification resulted in isolation of thymidine itself as the apparent bioactivity. As the unfractionated crude extract had activity which decreased cell viability and density, we proceeded to ascertain whether the low concentrations of thymidine present in chromatographic fractions had intrinsic and genuine effects on cell proliferation using bioassays that did not measure [^{3}H]thymidine incorporation. The effects of low concentrations of pure thymidine on 1) cell density, 2) cell viability, 3) distribution of cells in the cell cycle and 4) conversion of MTT to a coloured formazan compound was therefore investigated. Table 1 summarizes data showing that exogenous thymidine at submillimolar concentrations indeed had dose-dependent effects on all these indices of cell growth after a 48-hour treatment period.

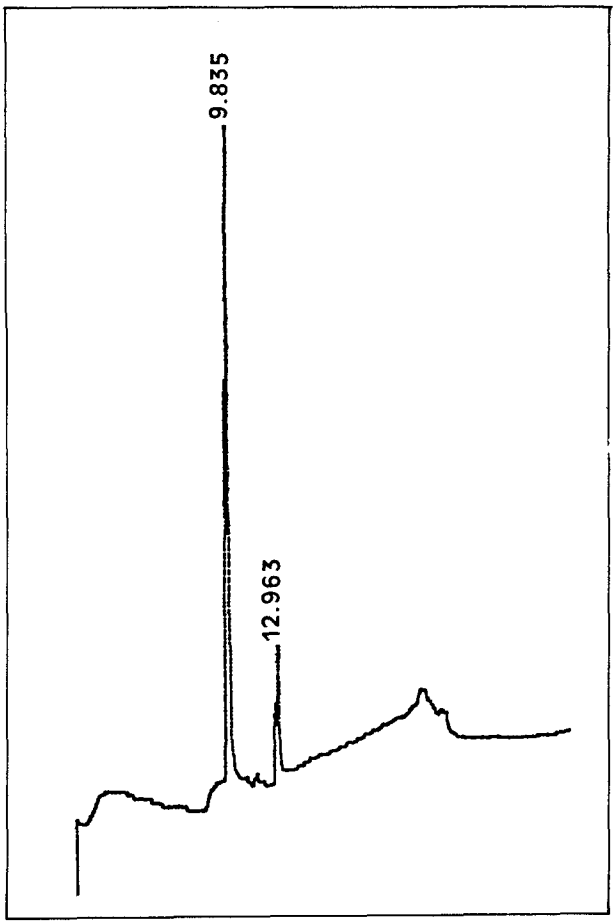

Figure 2. Fractionation of bioactivity by high performance chromatography. Details in text.

Thymidine had significant effects on cell proliferation at concentrations at least as low as 10^{-5} M. These effects were associated with a disturbance of cell cycle kinetics manifested by a significant increase in S phase cells (from 42.4% (SD 7.7%) in untreated cultures to 60.7% (SD 3.4%) in cultures treated with 10^{-5} M thymidine; $p < 0.03$) accompanied by a reciprocal and significant decrease in G_1 cells (from 47.8% (SD 6.5%) in untreated cultures to 28.7% (SD 1.9%) in cultures treated with 10^{-5} M thymidine; $p < 0.01$) (table 1). Thymidine at this concentration decreased cell density in a time-dependent manner. The difference was significant as early as 12 h after treatment and persisted for up to 48 h, at which time there was also a slight loss of viability (fig. 4).

These effects were thymidine-specific, as demonstrated by significant inhibition of MTT conversion by 10^{-5} M thymidine ($p < 0.001$ compared to control), whereas at the same concentration thymine, adenosine, guanosine, inosine and uridine were all ineffective (table 2).

Similar experiments showed no effect of 10^{-5} M adenosine, guanosine, inosine and uridine on EL4 cell density, cell viability and efficiency of MTT conversion (data not shown). In contrast, treatment with 10^{-5} M thymidine resulted in a significant decrease in cell density compared to untreated cells ($p < 0.0001$), associated with significant loss of viability ($p < 0.05$). Both these effects combined to give the lower efficiency of MTT conversion previously shown in table 1.

A potent inhibitor of facilitated nucleoside transport, nitrobenzylthioinosine (NBMPR)[6] was used to determine whether exogenous thymidine was acting extracellulary or intracellularly to produce the antigrowth effect on EL4 cells. Table 3 shows that the presence of 2×10^{-5} M NBMPR in EL4 cultures treated with 10^{-5} M thymidine for 24 h brought about virtually total reversal of the decline in cell density and completely normalized the distribution of cells in the cell cycle. This concentration of NBMPR also largely prevented cellular entry of exogenous thymidine (data not shown).

Table 1. Effects of thymidine on cell growth

Thymidine concentration	0	5×10^{-6} M	10^{-5} M	2×10^{-5} M	4×10^{-5} M	6×10^{-5} M
Cell density ($\times 10^{-6}$, cells/ml)	1.92 (0.04)	1.18* (0.01)	0.72* (0.03)	0.50* (0.04)	0.37* (0.02)	0.32* (0.01)
Cell viability (%)	89 (2.4)	87.7 (0.2)	74.5* (3.0)	49.5* (0.3)	23.9* (2.7)	16.6* (2.4)
MTT assay A_{570}	0.77 (0.03)	0.77 (0.01)	0.66* (0.02)	0.31* (0.02)	0.07* (0.004)	0.03* (0.008)
Cell cycle phases:						
G_1(%)	47.8 (6.5)	33.9 (0.4)	28.7* (1.9)	25.9* (3.6)		
S (%)	42.4 (7.7)	55.4 (0.4)	60.7* (3.4)	63.1* (6.8)		
G_2-M (%)	9.7 (1.2)	10.8 (0.1)	10.7 (1.6)	11.0 (3.9)		

EL4 cells were treated with the concentrations of thymidine stated for 48 h. Data are means and standard deviations (SD, within brackets) of triplicates. Values significantly different ($p < 0.05$) from controls without thymidine are indicated by an asterisk.

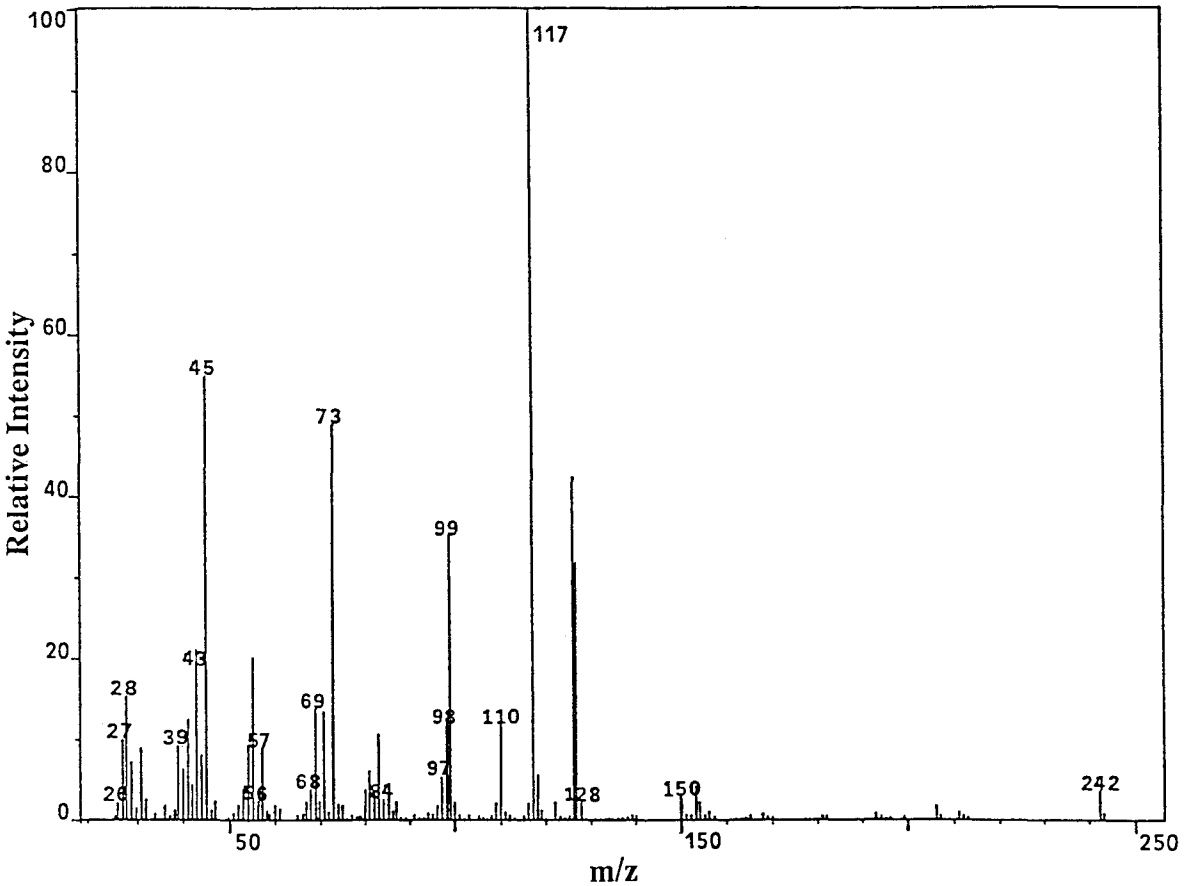

Figure 3. Mass spectrum of the bioactive metabolite (thymidine) isolated from *C. terminalis*.

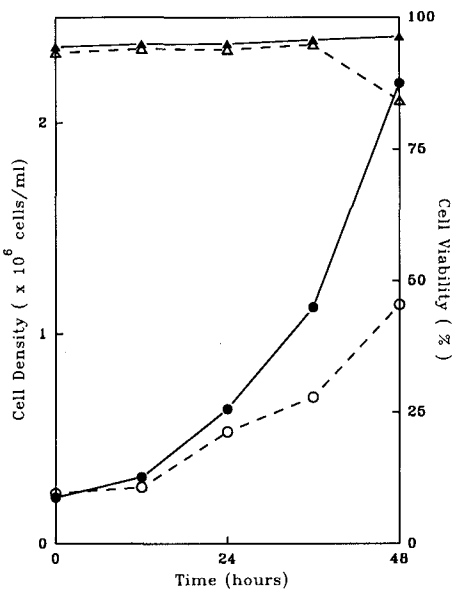

Figure 4. Time course of effect of thymidine on cell density (circles) and cell viability (triangles). Data points in filled symbols were from control cells (water alone) and in open circles from cells treated with 10^{-5} M thymidine.

Table 2. Specificity of effect of thymidine on cell growth

	Absorbance at 570 nm
Control	0.497 (0.014)
Adenosine	0.489 (0.034)
Guanosine	0.477 (0.029)
Inosine	0.507 (0.014)
Thymidine	0.342* (0.020)
Thymine	0.492 (0.012)
Uridine	0.474 (0.036)

EL4 cells were treated with 10^{-5} M of the compounds stated for 48 h. Effects of each on cell growth were assessed by the MTT bioassay[3]. Data are means and standard deviations (SD) of six replicate determinations. Only the value indicated by an asterisk is significantly different ($p < 0.0001$) from controls without thymidine.

Selectivity of target cell type in response to submillimolar concentrations of thymidine was investigated by treating two other cell lines with 10^{-5} M thymidine. As judged by the extent of MTT conversion to formazan (table 4), growth of K36 cells (a line established from a mouse lymphoma) was not decreased but growth of MCF7 cells (of human breast cancer origin) was inhibited.

Table 3. Reversal of thymidine effect by inhibitor of cellular uptake of thymidine

	Control	Thymidine	Thymidine + NBMPR
Cell density ($\times 10^{-6}$, cells/ml)	0.78 (0.017)	0.52* (0.040)	0.82* (0.012)
Cell viability (%)	91.3 (0.9)	83.3* (1.3)	89.0* (0.9)
MTT bioassay A_{570}	1.275 (0.01)	0.92* (0.01)	1.141* (0.05)

EL4 cells were treated for 24 h with 10^{-5} M thymidine alone or with the addition of 2×10^{-5} M NBMPR, an inhibitor of facilitated thymidine transport. Data are means and standard deviations (within brackets) of triplicate determinations for cell density and viability, and of six replicates for the MTT bioassay. Values significantly different from untreated controls ($p < 0.05$) are indicated by asterisks.

Discussion

Thymidine, a naturally occurring nucleoside, is known to inhibit cell replication. Until recently, however, the antiproliferative effect has been regarded as a pharmacological (rather than a physiological) effect because millimolar concentrations of thymidine were required to inhibit cell proliferation and cause cell death. This is several orders of magnitude higher than physiological thymidine concentrations[7] in plasma, which are in the range of 10^{-6}–10^{-7} M.

The target of thymidine's pharmacological effect on cell proliferation is ribonucleotide reductase, which catalyzes the reduction of ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs). Exogenous thymidine greatly expands the intracellular pool of dTTP, an allosteric inhibitor of ribonucleotide reductase. The consequent imbalance in dNTP pool sizes (decreased intracellular levels of deoxycytosine triphosphate and increased intracellular pools of deoxyadenosine and deoxyguanosine triphosphate)[8] impairs DNA replication and synchronizes cells in the S phase.

Thymidine also inhibits poly (ADP-ribose) polymerase, which catalyzes ADP-ribosylation of chromatin proteins. This form of protein modification is necessary for normal DNA excision-repair and its deficiency may contribute to cytotoxicity[9].

Thymidine in micromolar concentrations is present in the supernatants of cells in culture. The origin of extracellular thymidine has been ascribed to degradation of DNA of dead cells[10,11]. Cell death is not, however, the only source of extracellular thymidine in growth media, and more recent evidence has shown that thymidine is a secretory product of living cells[12–14]. This implies that thymidine may also have physiological growth-inhibitory effects.

The present studies show that leaves of *Cordyline terminalis* are a rich source of unmodified thymidine (about 110 mg was purified from 59 g of dried leaves) and extend what is currently known of the effects of low (submillimolar) concentrations of thymidine on cell replication. These data clearly show antiproliferative effects of thymidine in near-physiological concentrations, which have the following characteristics. Effects on cell replication and viability are highly specific to this nucleoside and are manifest within 12–24 h of treatment. Thymidine causes a characteristic perturbation of the cell cycle consistent with a block in S phase. It is of interest that the disorder in cell cycle distribution observed in these studies was also found in clinical trials of continuous infusions of thymidine for leukemia and lymphoma patients, in whose bone marrow an increase in S phase cells also occurred[15]. The antiproliferative action displays selectivity for tumour cell type and is prevented by NBMPR which inhibits facilitated diffusion of nucleosides. Differential sensitivity of cells to the cytotoxic effects of pharmacological (millimolar) concentrations of thymidine is known[16,17] and is probably due to quantitative and/or qualitative differences in ribonucleotide reductase. More recent observations also indicate the occurrence of differential sensitivity to submillimolar thymidine concentrations associated with thymidine kinase deficiency[13].

The above observations raise a number of potentially interesting questions. Given that submillimolar concentrations of thymidine are antiproliferative and that similar (or higher) concentrations may occur at local tissue levels, is thymidine a physiological inhibitor of cell proliferation? Although clinical trials of thymidine as a single chemotherapeutic agent of human malignancies were limited by side effects (gastrointestinal, haematological and neurological) and by the need to administer large volumes of intravenous fluids[7,15], the established antiproliferative effects of this natural compound should spur efforts to design thymidine analogues with a higher activity against cell proliferation and fewer adverse side effects. The effectiveness of one such analogue, azidothymidine, against human immunodeficiency virus suggests that other thymidine analogues could be as useful.

Table 4. Cell type selectivity of thymidine effect

K36 control	0.753 (0.022)	MCF7 control	0.622 (0.022)
K36 + 10^{-5} M thymidine	0.806 (0.018)	MCF7 + 10^{-5} M thymidine	0.506* (0.033)

The effect of treating K36 and MCF7 cells with 10^{-5} M thymidine for 48 h was assessed by the MTT bioassay. Data (absorbance at 570 nm) are means and standard deviations (within brackets) of 6 replicates. The value indicated by an asterisk is significantly lower than its control ($p < 0.001$).

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1 Burkhill, I. H., A Dictionary of the Economic Products of the Malay Peninsula, Vol. I, Crown Agents, London 1935.
2 Tang, B. L., Teo, C. C., Sim, K. Y., Ng, M. L., and Kon, O. L., Biochim. biophys. Acta *1014* (1989) 162.
3 Teo, C. C., Kon, O. L., Sim, K. Y., and Ng, S. C., J. Med. Chem. *35* (1992) 1330.
4 Hansen, M. B., Nielsen, S. E., and Berg, K., J. Immun. Methods *119* (1989) 203.
5 Bieman, K., and McCloskey, J. A., J. Am. chem. Soc. *84* (1963) 2005.
6 Sokoloski, J. A., Lee, C. W., Handschumacher, R. E., Nigam, A., and Sartorelli, A. C., Leuk. Res. *15* (1991) 1051.
7 O'Dwyer, P. J., King, S. A., Hoth, D. F., and Leyland-Jones B., Cancer Res. *47* (1987) 3911.
8 Bjursell, G., and Reichard, P., J. biol. Chem. *248* (1973) 3904.
9 Durkacz, B. W., Omidiji, O., Gray, D. A., and Shall, S., Nature *283* (1980) 593.
10 Opitz, H.-G., Neithammer, D., Jackson, R. C., Lemke, H., Huget, R., and Flad, H.-D., Cell. Immun. *18* (1975) 70.
11 Evans, R., and Booth, C. G., Cell. Immun. *26* (1976) 120.
12 Kasahara, T., and Shioiri-Nakano, K., J. Immun., *116* (1976) 1251.
13 Nakano, S., Koga, T., Ichinose, I., Yamada, H., and Niho, Y., Biochim. biophys. Acta *1094* (1991) 263.
14 Gehring, H., and Schroder, A., Biochem. biophys. Res. Commun. *177* (1991) 259.
15 Blumenreich, M. S., Woodcock, T. M., Andreeff, M., Hiddemann, W., Chou, T. C., Vale, K., O'Hehir, M., Clarkson, B. D., and Young, C. W., Cancer Res. *44* (1984) 2203.
16 Reynolds, E. C., Harris, A. W., and Finch, L. R., Biochim. biophys. Acta, *561* (1979) 110.
17 Lee, S. S., Giovanella, B. C., and Stehlin, J. S. Jr., J. Cell Physiol. *92* (1977) 401.