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ORIGINAL ARTICLE

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Thymidylate synthase inhibition induces S-phase arrest, biphasic mitochondrial alterations and caspase-dependent apoptosis in leukaemia cells

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Abstract In this study, the downstream effects of thymidylate synthase (TS) inhibition in L1210 (p53 mutant) and HL60 (p53 null) leukaemia cells were investigated. TS inhibition was induced by the specific TS inhibitor Thymitaq. Within 24 h, TS inhibition resulted in S-phase cell cycle arrest in both cell lines and subsequent apoptotic cell death as characterized by nuclear condensation, DNA fragmentation and the formation of apoptotic bodies. A biphasic hyper/hypopolarization of the mitochondrial membrane potential ($\Delta\Psi$m) was also observed. The mitochondrial permeability transition inhibitor, cyclosporin A, increased the baseline level of $\Delta\Psi$m in L1210 cells. However, along with bongkrekic acid, it did not influence the changes in $\Delta\Psi$m induced by TS inhibition in either cell line. In both cell lines the broad spectrum caspase inhibitor, zVAD.fmk as a single agent, induced a significant downward shift in the baseline of $\Delta\Psi$m. However, only in HL60 cells was this accompanied by a slight increase in cytotoxicity. In L1210 cells zVAD.fmk inhibited DNA fragmentation induced by Thymitaq but did not influence other cell cycle events (S-phase arrest) or the biphasic mitochondrial alterations, indicating caspase involvement downstream but not upstream of the mitochondria following TS inhibition. In HL60 cells, zVAD.fmk reduced the hyperpolarization of $\Delta\Psi$m observed with Thymitaq alone and failed to inhibit the increase in the sub-G_1 population induced by Thymitaq. Moreover, zVAD.fmk significantly increased the cell death response of these cells following TS inhibition. In conclusion, cell death induced by TS inhibition is mediated via the apoptotic pathway which clearly involves biphasic alterations in $\Delta\Psi$m. In L1210 cells, but not in HL60 cells, caspases function as the final executioner of apoptosis.

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Introduction

While the pharmacology of most anticancer agents is well understood, the biochemical mechanisms by which these drugs ultimately induce cell death are less-well defined. Many anticancer agents have been shown to mediate their cytotoxic effects via DNA damage and the subsequent induction of apoptosis [13, 19, 40]. In addition to these findings is the observation that anticancer drug resistance cannot be explained entirely by the changes upstream of the primary drug-target interaction but may also be mediated via alterations in the machinery governing apoptotic death [7, 8, 13, 39]. A greater understanding of drug-induced apoptotic cell death may permit manipulation of the key components of the apoptotic pathway in order to enhance the therapeutic index of anticancer drugs.

Thymidylate synthase (TS, EC 2.1.1.45) inhibitors are commonly used anticancer agents but the downstream effects of TS inhibition, which ultimately result in cancer cell death, are not clearly known. TS catalyses a critical step in the pathway of DNA synthesis by converting dUMP to dTMP by methylation using the co-substrate N5,N10-methylene tetrahydrofolate (CH_2-THF) as a methyl donor. This step is the only de novo source of dTMP, which is subsequently metabolized to dTTP exclusively for incorporation into DNA during synthesis and repair [20]. Thus, TS is a key regulatory enzyme during DNA synthesis. Lack of dTTP results in DNA damage and ultimately cell death, but the process(es) by which cell death occurs is (are) not clear. TS inhibitors such as fluorouracil, raltitrexed, and LY231514 play a pivotal role in anticancer treatment and are often the first-line treatment of many cancers [29].

Drug-induced apoptotic cell death begins with the primary drug-target interaction, which often results in DNA damage particularly in response to antimeta-

bolites and chelating agents [3, 29]. The detection of drug-induced damage and the decision to either repair the DNA or to induce cell death characterizes the next phase of this pathway [10], the outcome of which can vary with the type of drug used, the cell type under study and the expression of oncogenes [10, 27, 28]. While TS inhibition often induces apoptosis, the intermediate steps of this response have not been elucidated and are controversial [32], particularly with regard to the role of death receptors, caspases and the mitochondria [12, 17].

During apoptosis two mechanisms of mitochondrial participation have been described. One involves opening of the mitochondrial permeability transition (PT) pore complex, with organelle swelling and rupture of the outer membrane followed by release of apoptotic factors (cytochrome *c* and apoptosis-inducing factor) which initiate the cleavage of both upstream and downstream caspases [21, 31, 34]. The other relies on the direct release of these apoptotic factors without PT [12]. PT is characterized by a disruption of the inner transmembrane potential ($\Delta\Psi$m) that precedes the nuclear signs of apoptosis [22, 30] and is often inhibited by the tumour-promoter bcl-2 and agents such as cyclosporin A (CsA) and bongkrekic acid (BA) [6, 37, 43]. Bcl-2 is located in the mitochondria and is known to inhibit apoptosis induced by numerous anticancer agents [1, 11, 15, 16, 26, 36]. Cells overexpressing bcl-2 have also been shown to have a higher $\Delta\Psi$m than wild-type cells [14]. The mitochondria appear to function as the central amplifier of the apoptotic pathway irrespective of the trigger used to induce apoptosis. Furthermore, caspase activation has been reported to occur both upstream (death receptor/caspase 8/inducer) and downstream (caspase 9/executioner) of these mitochondrial alterations [12].

In the present investigation, we examined the mode of cell death, cell cycle alterations, mitochondrial perturbations and caspase activation induced by TS inhibition in L1210 and HL60 leukaemia cell lines. The quinazoline TS inhibitor, Thymitaq (nolatrexed, AG337, Zarix), was specifically chosen in this study over the more conventional agents since it is a noncompetitive, direct and specific inhibitor of TS, with no requirement for active membrane transport or intracellular activation [18, 41]. The lack of intermediate steps enabled us to pharmacologically manipulate the downstream cell death pathway without the concern of interfering with the primary drug-target interaction.

Materials and methods

Tissue culture and stock solutions

The following stock solutions were prepared and stored at –20 °C. Thymitaq (Zarix, King of Prussia, Pa.) 5 m*M* in distilled water, propidium iodide (Sigma, St Louis, Mo.) 400 μg/ml in phosphate buffered saline (PBS), proteinase K (Sigma) 20 mg/ml in PBS, RNase A (Sigma) 5 mg/ml in distilled water, CsA (Sigma) 1 m*M* in PBS, rhodamine-123 50 m*M* in DMSO and zVAD.fmk (benzoyl-oxycarbonyl-VAD-fluoromethylketone; Biomol) 5 μ*M* in DMSO. The cell lines L1210 (murine lymphocytic leukemia, p53 mutant) and HL60 (human promyelocytic leukemia, p53 null) were maintained at 37 °C in an atmosphere containing 5% CO_2 in RPMI-1640 (Trace Biosciences, Australia) supplemented with 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (4 m*M*). The doubling times were 12–14 h for L1210 cells and 24–26 h for HL60 cells, and the cells were passaged every 3–5 days. Both cell lines were routinely tested and found to be mycoplasma-free.

Cytotoxicity assay

Cells in logarithmic growth were transferred to 96-well round bottomed plates. Cytotoxicity was determined by plating cells in triplicate in 100 μl medium at a density of 2,500 and 1,000 cells/well (L1210) and 10,000 and 5,000 cells/well (HL60) for 48 and 72 h, respectively. On day 0 (24 h after plating) when the cells were in logarithmic growth, 100 μl medium with or without Thymitaq 1×10^{-4} to 5×10^{-8} *M* was added to each well. After drug exposure growth-inhibitory effects were evaluated using the MTT [3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay and absorbance was read at 570 nm [2]. The IC_{50} was the drug concentration at which cell growth was inhibited by 50% based on the difference between the optical density values on day 0 and those at the end of drug exposure [5]. Similar cytotoxicity assays were done using 24-h Thymitaq treatment to determine appropriate concentrations for mechanistic studies.

Detection of apoptosis by Hoechst staining

Logarithmic phase cells were plated at 5,000 cells/well (L1210) and 12,500 cells/well (HL60) in 24-well tissue culture plates and treated 24 h later with Thymitaq. The cells were permeabilized 24 h after drug exposure using a mild hypotonic solution (1:1, growth medium/distilled water, 10 min), fixed with methanol/acetic acid (3:1, 10 min) and stained with Hoechst 33258 (Sigma, 10 μg/ml, 10 min). The blue fluorescence of the dye bound to DNA was detected using a fluorescence microscope. Apoptotic cells stained brightly and displayed condensed and fragmented nuclei. Normal cells showed an even distribution of the stain throughout the nucleus with flocculated chromatin.

DNA fragmentation analysis

DNA fragmentation was evaluated by gel electrophoresis analysis. Cells ($2–5 \times 10^6$) were pelleted, washed, resuspended in 500 μl lysis buffer (0.1 m*M* EDTA, 10 m*M* Tris-HCl, pH 8.0, 0.5% SDS) for 1 h at 37 °C. Proteinase K digestion (4 μg/ml, 3 h, 50 °C) was followed by phenol chloroform DNA extraction [42]. DNA was electrophoresed (10 μg, 75 V, 3 h) through a 1.8% agarose gel, stained with ethidium bromide (0.5 μg/ml, 30 min), visualized by UV irradiation and photographed with a Polaroid camera. The fragmentation of DNA into multiples of 180–200 bp produces a distinct ladder pattern on the agarose gel.

Mitochondrial membrane potential ($\Delta\Psi$m)

Tumour cells were cultured in 25-cm^2 culture flasks and allowed to reach exponential growth for 24 h prior to treatment. The cells were harvested 24 h after drug treatment, and assessed for changes in $\Delta\Psi$m as measured by uptake of the lipophilic cation rhodamine-123 into mitochondria [4]. Briefly, the cells were pelleted by centrifugation at 300–500 g for 5 min at room temperature and resuspended in 2 ml PBS. An aliquot (1 ml) of the cell sample was kept for cell cycle analysis. The remaining cells were incubated with 1 ml rhodamine-123 (20 μg/ml) for 30 min at room temperature and washed with PBS. The samples (2×10^4 events) were analysed for fluorescence (FL1 detector, filter 430/30 nm bandpass) using a FACScan (Becton-Dickinson, Sunnyvale, Calif.). Histograms were analysed using Cell Quest software, and compared with histograms

of control untreated cells to calculate the percentages of treated cells with a low, normal and high $\Delta\Psi$m.

Cell cycle analysis

Tumour cells were cultured and treated with or without Thymitaq as detailed above in the $\Delta\Psi$m assay. The 1 ml cell aliquot remaining from the $\Delta\Psi$m assay was pelleted at 300–500 *g* for 5 min and fixed in 70% ethanol and stored at –20 °C until analysis (1–2 weeks). The pellet was incubated in 1 ml PBS containing propidium iodide (40 μg/ml) and RNase (200 μg/ml) for at least 30 min at room temperature. The samples (2×10^4 events) were analysed for fluorescence (FL2 detector, filter 575/30 nm bandpass) using a FACScan (Becton Dickinson). Cell cycle distribution was assessed using Cell Quest software.

Statistical analysis

The results was assessed by analysis of variance. Percentage data were arcsine-transformed before analysis [35]. All values presented in the figures are means ± SEM of the nontransformed data. Data were analysed using Statistica (Ver. 4.5; Statsoft, Tulsa, Okla.).

Results

Thymitaq-induced cytotoxicity and apoptotic cell death

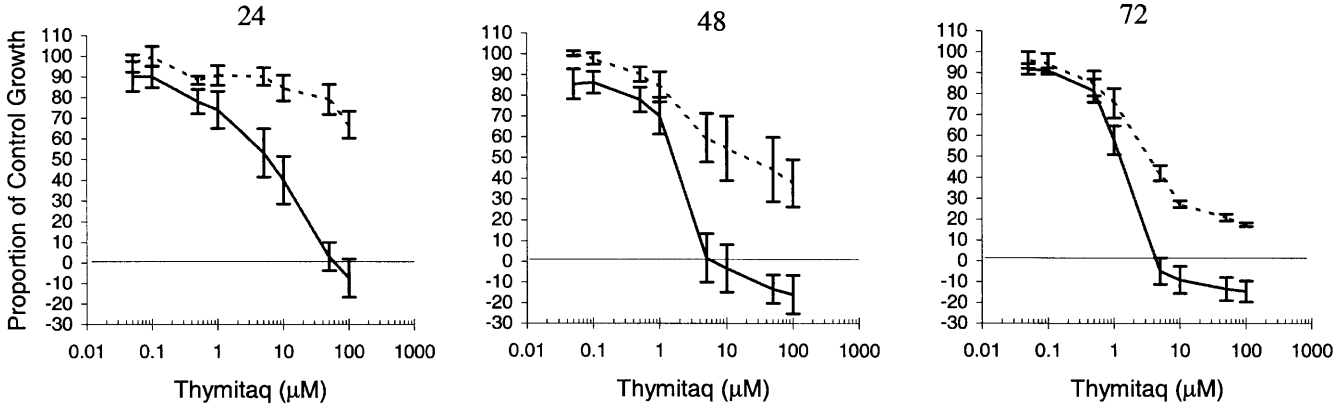
Thymitaq was cytotoxic in L1210 and HL60 leukaemia cell lines producing IC_{50} values of 3.6 ± 0.6 μ*M* and 1.3 ± 0.3 μ*M*, respectively, after continuous exposure for 72 h (Fig. 1). Dead cells were visible following treatment with Thymitaq and the concentration-response curve for HL60 cells fell below the baseline cell number, confirming that Thymitaq was cytotoxic and not just cytostatic in these cells. Exposure of the cells to Thymitaq for shorter times (24 and 48 h) resulted in higher IC_{50} values. Thymitaq (10 and 50 μ*M*) also induced the classical morphological changes of the execution phase of apoptotic cell death, including cell surface membrane blebbing and the formation of membrane-bound apoptotic bodies, which were evident 24 h after exposure to the drug (Fig. 2). Condensed and fragmented nuclei (Fig. 3) together with the fragmentation of DNA into multiples of 200 bp (Fig. 4) were also observed in Thymitaq-treated cells (10 and 50 μ*M*) after 24 h, further confirming the ability of Thymitaq to induce apoptotic cell death. The results of this study clearly show that Thymitaq-induced cell death occurred by apoptosis in these leukaemia cell lines.

Cell cycle analysis

Table 1 and Fig. 5 show the cell cycle distribution of asynchronous L1210 and HL60 cells following exposure to various concentrations of Thymitaq for 12 and 24 h. The distribution of untreated cycling cells in both cell lines was similar with a sub-G_1 (apoptotic) population of 2–4%, a G_1 population of 42–50%, an S-phase population of 20–27% and a G_2 + M population of 26–29%. In L1210 cells minor nonsignificant alterations in the cell cycle distribution were observed after 12 h of treatment with Thymitaq (10 and 50 μ*M*) when compared with untreated control cells. However, after 24 h a significant ($P < 0.001$) concentration-dependent increase in the proportion of sub-G_1 cells was evident which was accompanied by a significant ($P < 0.001$) concentration-dependent decrease in the proportion of cells in G_2 + M. Interestingly at this time-point, 5 μ*M* Thymitaq induced a significant ($P < 0.001$) increase in the proportion of cells in S-phase due to S-phase arrest (Fig. 5).

A higher dose of Thymitaq (10 μ*M*) also induced S-phase arrest ($P < 0.05$) but at an earlier point in S-phase as indicated by the broadening of the histogram at the G_1/S interface (Fig. 5). In contrast, Thymitaq at 50 μ*M* significantly ($P < 0.01$) reduced the S-phase component and failed to produce any sign of early to mid S-phase cell cycle arrest (Fig. 5). Collectively, the data show that in L1210 cells, low concentrations of Thymitaq for 24 h produced a cell population in which some cells were undergoing S-phase cell cycle arrest while others were undergoing DNA degradation. At higher concentrations, S-phase cell cycle arrest was not apparent and an even larger proportion of the cell population was undergoing DNA degradation.

Fig. 1 Concentration response curves (MTT assay) of L1210 (- - -) and HL60 (—) cells 24, 48 and 72 h after continuous exposure to various concentrations (0.05–100 μ*M*) of Thymitaq. Values are the mean ± SEM of 3–4 analyses



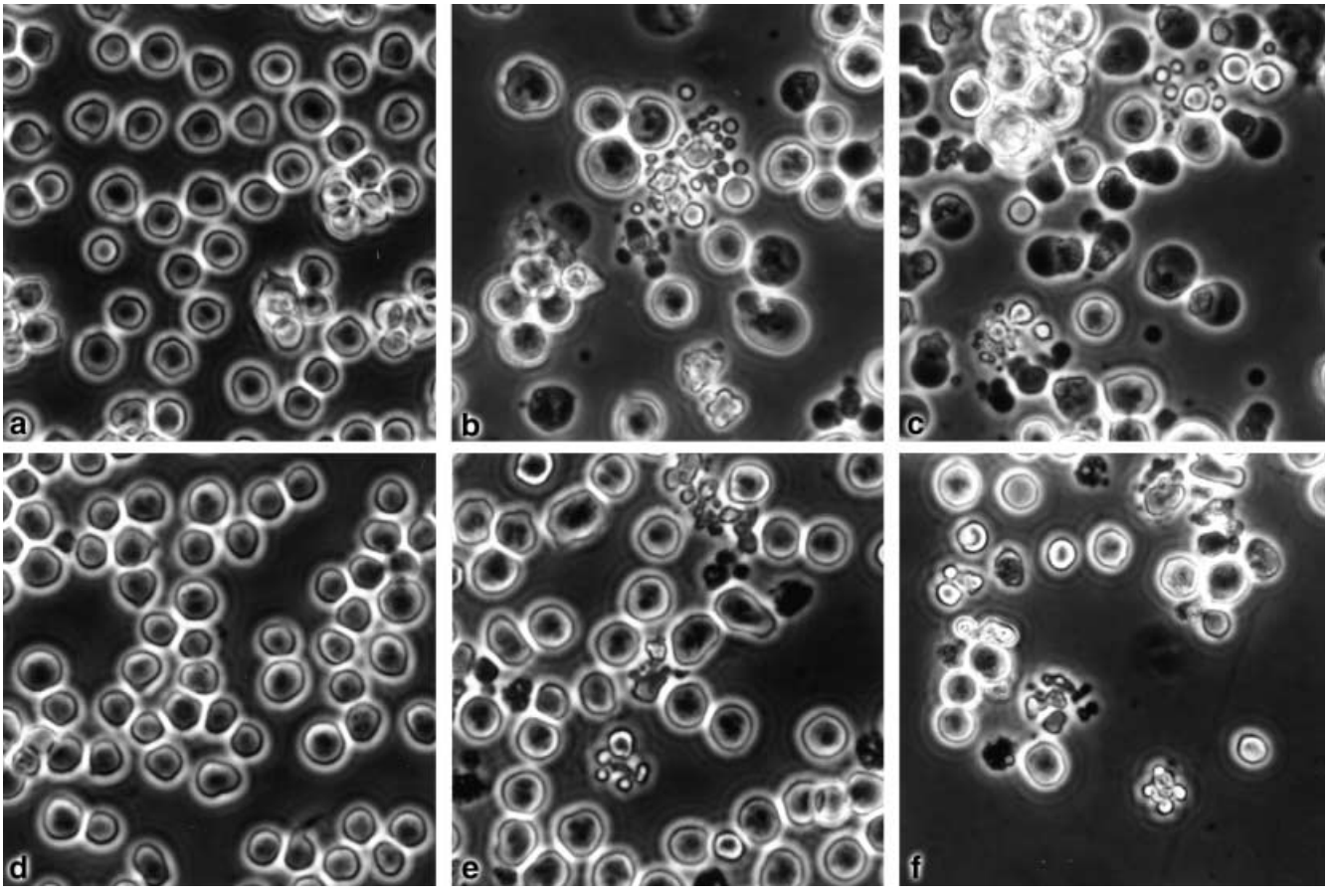


Fig. 2a–f Inverted microscopy of unstained L1210 cells (**a–c**) and HL60 cells (**d–f**) 24 h after exposure to Thymitaq (**a**,**d** controls; **b**,**e** 10 μ*M*; **c**,**f** 50 μ*M*) (×3400)

Significant cell cycle alterations in response to Thymitaq were observed in HL60 cells earlier than in L1210 cells. After only 12 h of treatment, concentrations of 10 and 50 μ*M* produced a significant ($P < 0.05$, $P < 0.001$) increase in the proportion of sub-G_1 cells, which was accompanied by a decrease ($P < 0.01$) in the G_2 + M population. The S-phase population also significantly ($P < 0.05$) declined at the higher concentration. No S-phase cell cycle arrest was apparent at the 12-h time-point. As in L1210 cells, in HL60 cells, 24 h of treatment with Thymitaq at 5, 10 and 50 μ*M* produced a significant concentration-dependent increase in the sub-G_1 cell population, with an accompanying significant decrease in the G_2 + M population. S-phase arrest was seen in HL60 cells 24 h after treatment with Thymitaq at 5 and 10 μ*M*, but not at 50 μ*M*. Collectively, Thymitaq induced similar trends in cell cycle distribution in the two cell lines tested with changes observed earlier in HL60 cells. In both cell lines an S-phase cell cycle arrest was clearly evident at low concentrations after 24 h but not after 12 h. Furthermore, early or mid S-phase arrest was not apparent in response to a higher concentration of Thymitaq in these cells.

Mitochondrial perturbations

Table 1 and Fig. 6 show the mitochondrial membrane potential of asynchronous L1210 and HL60 cells after 12 and 24 h exposure to various concentrations of Thymitaq. After 12 h of treatment with Thymitaq (10 and 50 μ*M*) L1210 cells showed no significant alterations in the ΔΨm when compared with the untreated control cells (Table 1). However, 24 h of treatment induced a significant ($P < 0.01$–0.001) concentration-related increase in the proportion of cells with a low ΔΨm. Interestingly, the proportion of cells with a normal ΔΨm declined in response to Thymitaq, but not in a concentration-related manner. Concomitantly with these changes, the proportion of cells with a hyperpolarized ΔΨm increased following Thymitaq treatment with the greatest response at the lower concentrations (5 and 10 μ*M*). Collectively, the data show that in L1210 cells, treatment for 24 h with low concentrations of Thymitaq caused a biphasic hyper/hypopolarization of ΔΨm. At higher concentrations a similar response was observed, but a hypopolarized ΔΨm profile predominated.

In HL60 cells, after 12 h of treatment with Thymitaq (10 and 50 μ*M*), a significant increase in the proportion of cells with a hypopolarized ΔΨm was observed concomitantly with a significant decline in the proportion of cells with a normal ΔΨm. This is in contrast to the effect in L1210 cells which did not show any mitochondrial

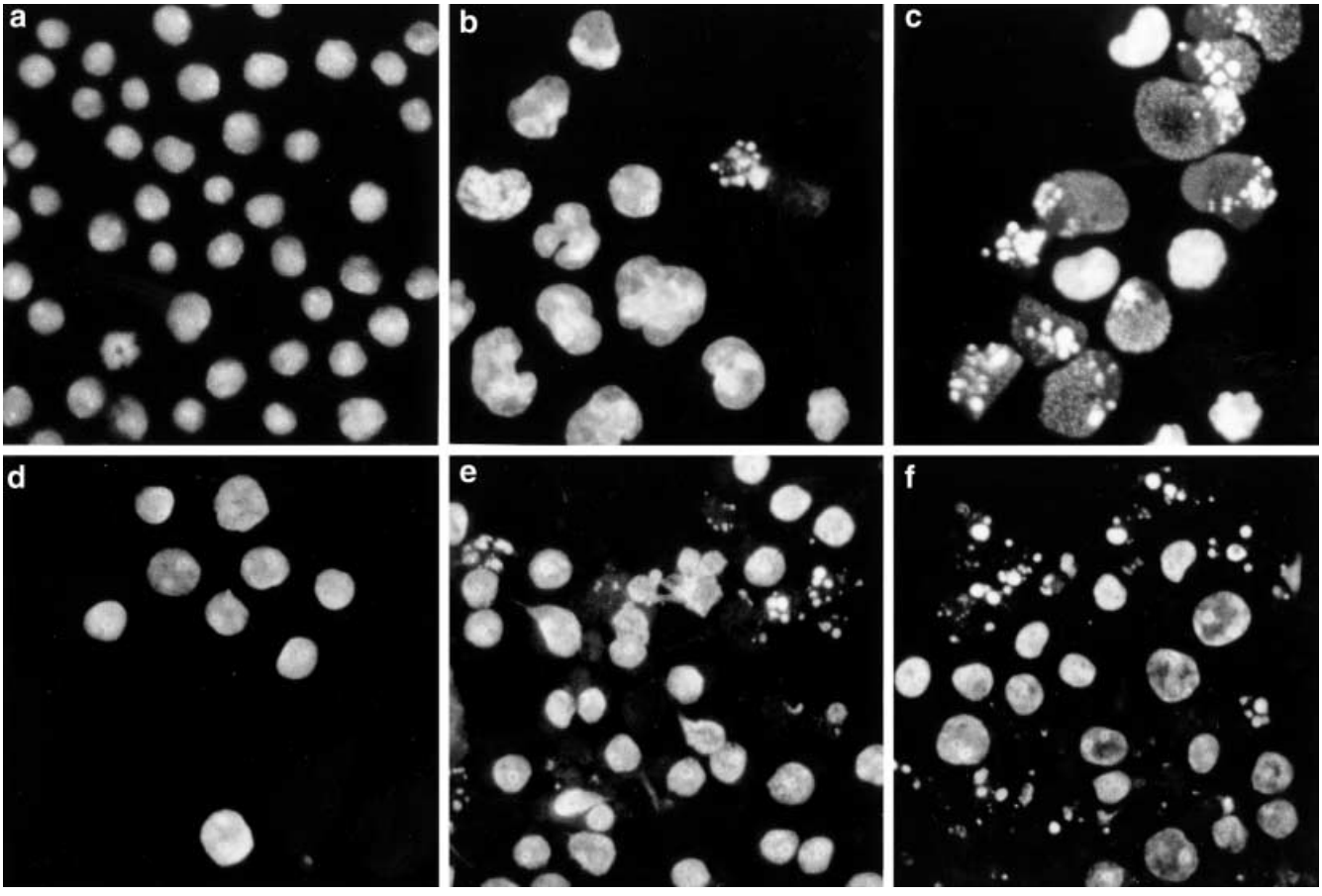


Fig. 3a–f Fluorescent staining of nuclei from L1210 cells (**a–c**) and HL60 (**d–f**) 24 h after exposure to Thymitaq (**a**,**d** controls; **b**,**e** 10 μ*M*; **c**,**f** 50 μ*M*) (×4800)

perturbations after 12 h of exposure to Thymitaq. However, the hyper-/hypopolarization of the ΔΨm in HL60 cells in response to Thymitaq after 24 h was essentially the same, in terms of the trend and magnitude, as that in L1210 cells (Table 1).

Permeability transition

The effects of the PT inhibitors CsA and BA on the mitochondrial response were also examined. CsA alone increased the baseline of ΔΨm in L1210 cells (Table 2, Fig. 7). CsA plus Thymitaq induced the same trends in ΔΨm as Thymitaq alone, but the proportion of cells with a hyperpolarized ΔΨm was much greater. Therefore, CsA accentuated the increase in ΔΨm induced by Thymitaq alone but otherwise did not affect Thymitaq-induced changes in ΔΨm. BA induced a similar response to that observed with CsA, but the increased baseline level of ΔΨm was only slight and not statistically significant. In HL60 cells CsA and BA had no effect on baseline ΔΨm, or Thymitaq-induced changes in ΔΨm.

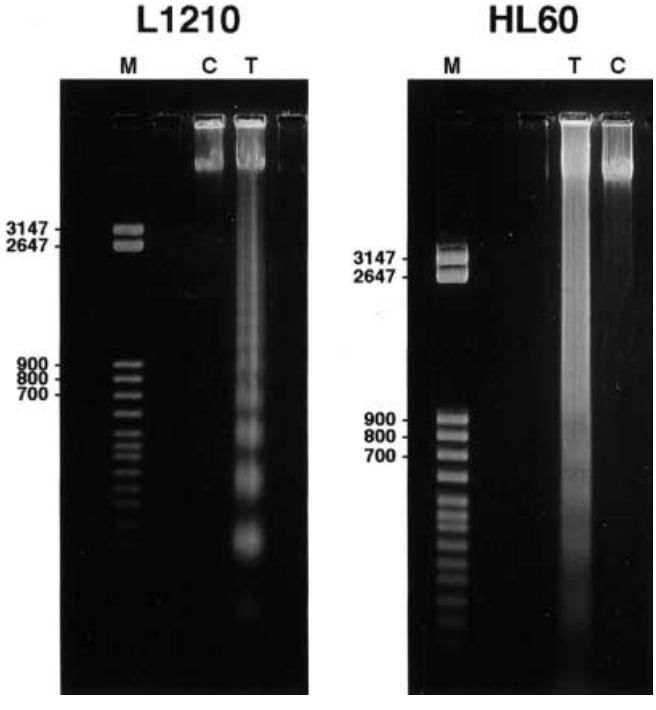


Fig. 4 DNA electrophoresis of L1210 and HL60 cells 24 h after exposure to 10 μ*M* Thymitaq (10 μg DNA added to each lane) (*C* untreated control cells; *T* Thymitaq-treated cells; *M* marker, 50 bp ladder)

Table 1 Cell cycle distribution and mitochondrial membrane potential of asynchronous leukaemia cells after continuous exposure to Thymitaq. Values are the means ± SEM from five or six independent experiments

Cell line	Exposure time (h)	Thymitaq (μ*M*)	Cell cycle distribution				ΔΨm		
			Sub-G_1	G_1	S	G_2 + M	Low	Normal	High
L1210	12	0	4 ± 1.7	47 ± 1.7	23 ± 4.7	26 ± 1.3	6 ± 0.7	90 ± 1.5	2 ± 1.2
		10	5 ± 1.5	53 ± 2.4	20 ± 3.1	21 ± 3.7	5 ± 1.4	91 ± 2.6	2 ± 1.3
		50	6 ± 1.7	52 ± 0.7	19 ± 3.0	21 ± 2.6	8 ± 2.0	89 ± 2.6	2 ± 0.8
	24	0	2 ± 0.4	42 ± 1.9	27 ± 1.3	29 ± 1.2	3 ± 0.6	94 ± 0.9	3 ± 0.4
		5	11 ± 2.0***	13 ± 2.6***	57 ± 3.2***	19 ± 1.0***	10 ± 2.1**	57 ± 3.8***	31 ± 3.9***
		10	21 ± 4.3***	20 ± 4.1***	42 ± 5.0*	16 ± 1.6***	14 ± 3.2**	60 ± 4.1***	25 ± 3.6***
		50	46 ± 8.5***	24 ± 6.0**	18 ± 3.8*	10 ± 2.0***	19 ± 5.7***	64 ± 6.6***	16 ± 4.7***
HL60	12	0	4 ± 0.5	50 ± 2.2	20 ± 1.0	27 ± 1.5	12 ± 4.3	86 ± 4.6	1 ± 0.1
		10	17 ± 5.9*	49 ± 0.8	16 ± 2.8	13 ± 0.9**	21 ± 5.0	76 ± 3.8	3 ± 1.0
		50	24 ± 2.7***	54 ± 3.0	13 ± 1.2*	9 ± 1.5**	26 ± 2.1*	71 ± 3.0*	2 ± 0.7
	24	0	2 ± 0.8	47 ± 1.1	25 ± 0.8	26 ± 0.9	5 ± 1.9	93 ± 1.9	1 ± 0.2
		5	13 ± 1.8***	23 ± 2.4***	49 ± 1.4***	15 ± 0.7***	15 ± 3.5**	71 ± 4.4***	13 ± 1.7***
		10	21 ± 3.2***	32 ± 4.8**	34 ± 2.6**	14 ± 1.2***	19 ± 3.1***	67 ± 3.9***	13 ± 1.6***
		50	41 ± 3.3***	39 ± 3.2*	12 ± 0.8***	6 ± 0.7***	28 ± 5.0***	66 ± 5.1***	5 ± 0.7***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs control (zero dose of Thymitaq)

Caspase activation

The broad-spectrum caspase inhibitor (zVAD.fmk) as a single agent, induced a significant downward shift in the baseline of ΔΨm (Table 3). However, only in HL60 cells was this accompanied by a slight increase in cytotoxicity. In L1210 cells zVAD.fmk significantly reduced ($P < 0.05$) the proportion of sub-G_1 cells in response to Thymitaq. However, it did not influence the induction of S-phase cell cycle arrest, or the biphasic hyper-/hypopolarization of ΔΨm induced by Thymitaq, indicating caspase activation only downstream of the mitochondria. In HL60 cells, zVAD.fmk inhibited the hyperpolarization of ΔΨm observed with Thymitaq alone but failed to inhibit the increase in the sub-G_1 population induced by Thymitaq. Moreover, zVAD.fmk significantly increased the cell death response of HL60 cells following TS inhibition.

Discussion

This study confirmed the cytotoxicity of Thymitaq in L1210 and HL60 cells with IC_{50} values similar to those reported by others [9, 33, 38, 41]. Cytotoxicity was both concentration- and time-dependent consistent with an inhibitor of DNA synthesis. The IC_{50} values for these cell lines were similar after a 72-h exposure, but the concentration-response curves differed substantially with shorter exposure times indicating different time dependencies. For example, Thymitaq was 20 times more potent in HL60 than in L1210 cells with a 24-h exposure, but only 4 times more potent with a 72-h exposure (Fig. 1). This difference cannot be explained by differences in cellular proliferation, since the L1210 cells grow at a substantially faster rate than HL60 cells and would therefore be expected to be more sensitive.

Several possible explanations can be suggested for the differing sensitivity of these two cell lines to Thymitaq. Differences in TS levels are unlikely to be the reason, since Estlin et al. [9] have reported that the sensitivity of a range of colon and leukaemia cell lines (including HL60 cells) to Thymitaq is not related to TS activity, or TS protein or mRNA levels. A difference in p53 status is also a possible explanation. O'Connor et al. [28] conclude that cells that are p53 mutant tend to exhibit less growth inhibition than wild-type p53 cell lines when treated with anticancer agents including TS inhibitors. We have confirmed that HL60 cells are p53 null while L1210 cells are p53 mutant, by the absence of a G_1-block following γ-irradiation (data not shown) [28]. Thus, p53 does not appear necessary for Thymitaq-induced cytotoxicity in these leukaemia cell lines. A similar finding has been observed in solid tumour cells in which antifolate-induced S-phase arrest and DNA damage is not caused by p53-mediated G_1-G_2 checkpoint mechanisms or p21-induced inactivation of the DNA-replicating machinery in SW480 (p53 mutant) and HCT-8 (p53 wild-type) colon cells [25]. Therefore, since neither p53 status nor proliferation rate explains the differences in TS inhibition-induced cytotoxicity in these cells lines, other factors are clearly involved in coordinating drug-induced cytotoxicity.

TS inhibition following Thymitaq treatment in leukaemia cells resulted in concentration-dependent apoptosis, as shown by the morphological and DNA changes (Figs. 2, 3 and 4). Such changes have been observed in response to other antimetabolites, including other TS inhibitors [32]. The response of L1210 and HL60 cells to low concentrations of Thymitaq was quite different to that induced by higher levels. The predominant response to low concentrations of Thymitaq for 24 h was S-phase cell cycle arrest and a biphasic hypo-/hyperpolarization of the ΔΨm with the latter predominating. This mid-S-phase cell cycle arrest was possibly related to a

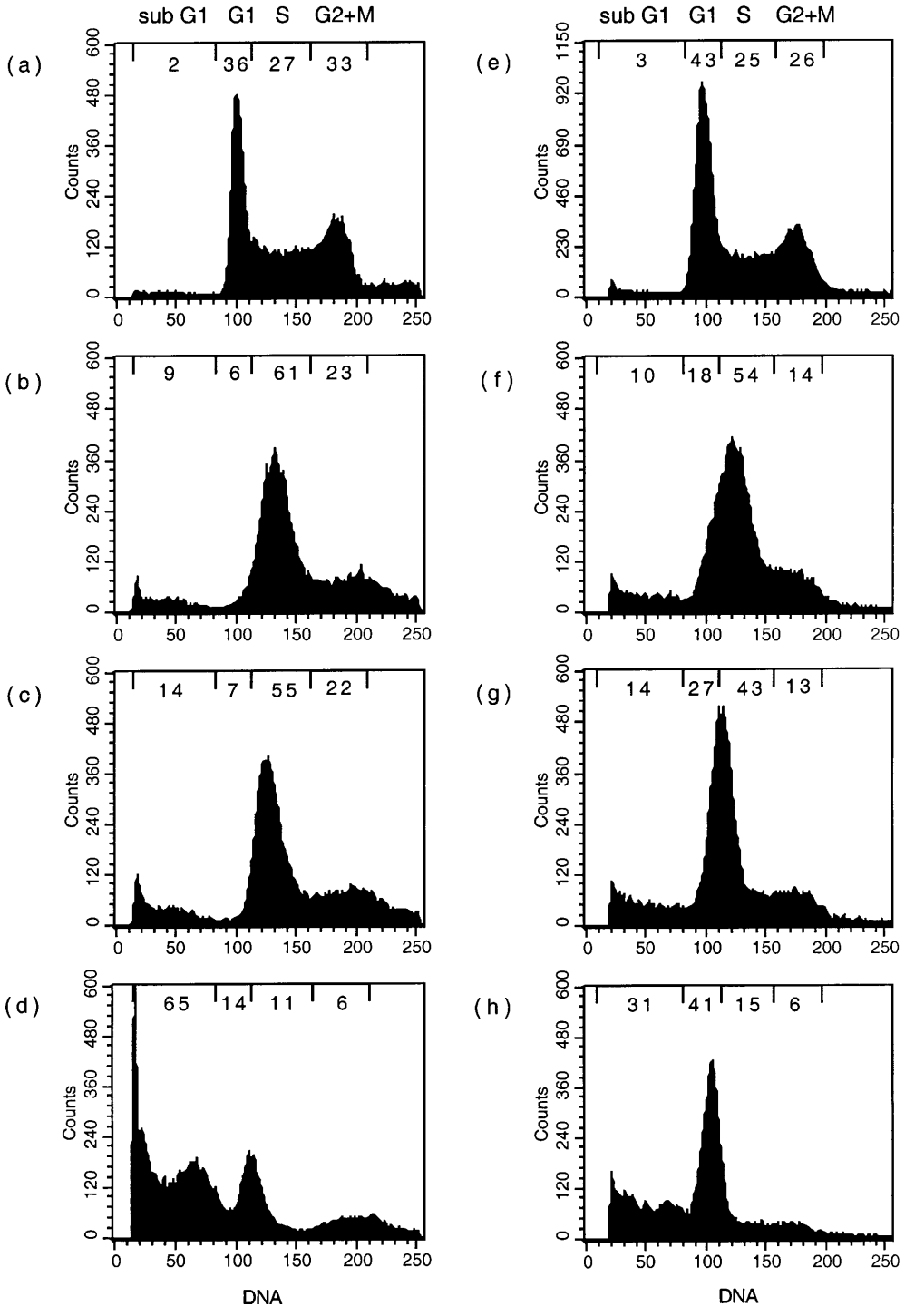


Fig. 5a–h Cell cycle distribution (percentage of total) of asynchronous L1210 cells (**a–d**) and HL60 cells (**e–h**) 24 h after continuous exposure to Thymitaq (**a**,**e** controls; **b**,**f** 5 μM; **c**,**g** 10 μM; **d**,**h** 50 μM). Each series of histograms is of one replicate data set and is representative of the typical response observed following Thymitaq treatment

diminished supply of DNA precursor components. The association between hyperpolarization of $\Delta\Psi$m and S-phase arrest has not previously been recognized, and may reflect the increased stress that the cell and its mitochondria experience under such conditions.

Exposure of cells to higher concentrations of Thymitaq did not produce an obvious mid-S-phase arrest, but the cells may have arrested at the G_1/S interface. Notwithstanding this, the predominant response to increased TS inhibition was an increase in the sub-G_1 population and hypopolarization of the $\Delta\Psi$m. Therefore, in response to the increased depletion of dTMP following treatment with the higher concentration of Thymitaq, cell death may have been directly induced rather than the cells undergoing any form of cell cycle arrest. We did not observe S-phase arrest at earlier time-points using high concentrations, so it seems more likely that the extent of the reduction in the dTMP pool induced by profound TS inhibition was so great that S-phase arrest did not occur and apoptosis was

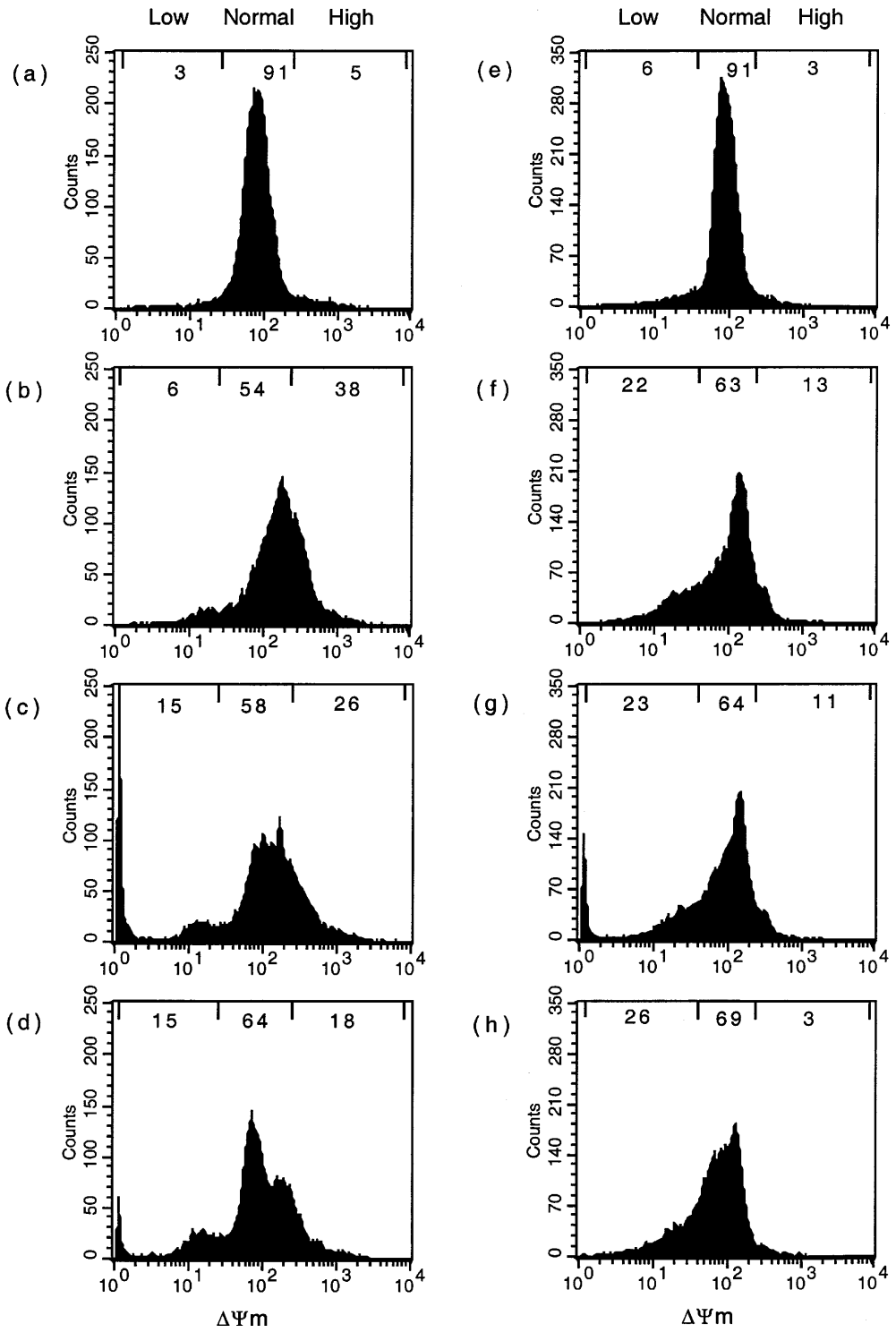


Fig. 6a–h Mitochondrial membrane potential (ΔΨm, percentage of total) in asynchronous L1210 cells (**a–d**) and HL60 cells (**e–h**) 24 h after continuous exposure to Thymitaq (**a**,**e** controls; **b**,**f** 5 μ*M*; **c**,**g** 10 μ*M*; **d**,**h** 50 μ*M*). Each series of histograms is of one replicate data set and is representative of the typical response observed following Thymitaq treatment

induced directly. This proposal is supported by Martin et al. [24] who suggested that the biochemical cascade of chemotherapy-induced apoptosis is dependent on the severity of the initial insult: modest damage induces arrest while massive damage induces apoptotic cell death. Such a phenomenon has also been observed following exposure of leukaemia cells to Ara-C and anthracyclines [40].

The Thymitaq-induced hypopolarization of ΔΨm is similar to mitochondrial changes observed during apoptosis induced by other mechanisms [12] and it is believed to be caused by the opening of the mitochondrial PT pore, disrupting the integrity of the inner membrane of the mitochondria thus leading to loss of ionic potential across the membrane and inhibition of ATP production [12]. This disruption also causes the matrix of

Table 2 Mitochondrial membrane potential (ΔΨm) in asynchronous leukaemia cells 24 h after continuous exposure to Thymitaq and cyclosporin A or bongkrekic acid. Values are the means ± SEM from three to five independent experiments

Cell line	Thymitaq (μ*M*)	CsA (μ*M*)	BA (μ*M*)	ΔΨm (percentage of total cells)		
				Low	Normal	High
L1210	0			4 ± 0.7	93 ± 1.2	3 ± 0.5
	5			12 ± 2.6	55 ± 4.7	32 ± 4.8
	10			16 ± 4.1	57 ± 5.3	25 ± 4.4
	50			25 ± 7.8	61 ± 8.9	12 ± 3.4
	0	1		3 ± 0.6	82 ± 1.8**	14 ± 4.8**
	5	1		12 ± 4.6	36 ± 7.4*	45 ± 3.8
	10	1		26 ± 6.4	37 ± 6.8*	34 ± 5.1
	50	1		33 ± 11	43 ± 4.1	22 ± 5.8
	0		50	3 ± 0.7	87 ± 6.1	10 ± 4.5
	5		50	6 ± 2.0	48 ± 9.0	44 ± 5.8
	10		50	9 ± 4.2	59 ± 7.1	30 ± 4.6
	50		50	20 ± 2.3	65 ± 5.3	14 ± 3.5
HL60	0			7 ± 1.8	91 ± 1.7	2 ± 0.2
	5			19 ± 3.2	61 ± 4.2	18 ± 3.1
	10			21 ± 3.1	62 ± 3.7	15 ± 1.8
	50			29 ± 5.6	65 ± 5.3	5 ± 0.5
	0	1		10 ± 3.4	87 ± 3.0	2 ± 0.7
	5	1		21 ± 5.2	65 ± 6.5	12 ± 4.4
	10	1		27 ± 5.2	58 ± 3.7	13 ± 3.5
	50	1		45 ± 12	51 ± 12	3 ± 1.0
	0		50	4 ± 1.5	94 ± 1.5	2 ± 0.3
	5		50	9 ± 1.5	68 ± 9.2	21 ± 9.3
	10		50	14 ± 2.6	73 ± 5.0	12 ± 3.7
	50		50	18 ± 1.9	75 ± 2.3	6 ± 2.1

* $P < 0.05$, ** $P < 0.01$, vs the corresponding dose of Thymitaq alone

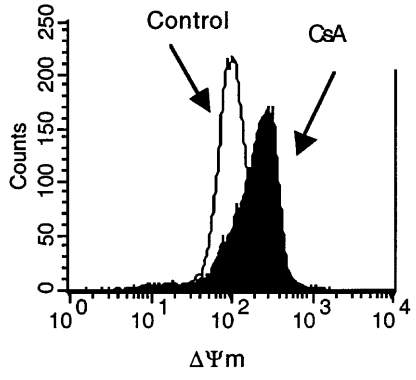


Fig. 7 Mitochondrial membrane potential (ΔΨm) in asynchronous L1210 cells (*control* untreated, *CsA* treated with 1 μ*M* cyclosporin A)

the inner membrane to expand and swell, leading to the rupture of the outer membrane and the release of mitochondrial components into the cytoplasm. The hyperpolarization we observed may have been due to mitochondrial swelling by mechanisms unknown, or may have been driven by respiration, as a result of enhanced oxidative phosphorylation and increased ATP production. In either case, an increase in ΔΨm would precede a decline in ΔΨm. This biphasic mitochondrial phenomenon was clearly observed following TS inhibition as the ΔΨm increased in response to the initial insult and declined in response to a longer and/or greater insult.

CsA and BA have been shown to inhibit PT and subsequent apoptotic cell death induced by various agents including dexamethasone, etoposide and irradiation [22]. They are believed to mediate their effects via the adenine nucleotide translocator (ANT), a constituent of the mitochondrial PT pore [12, 22]. However, in this study the collapse in ΔΨm induced by TS inhibition was not affected by either of these agents, suggesting that the ANT is not involved in apoptosis induced via TS inhibition. Clearly, the ANT is active in these cells as indicated by an increased baseline level of ΔΨm following exposure to these agents. Caspases, particularly caspase 9, have been described as the executioners of the apoptotic cell death pathway and in L1210 cells this role appears to hold true. However, in HL60 cells caspases do not function as the central executioner of apoptosis since zVAD.fmk did not prevent but in fact increased cell death. In this context, other researchers have observed the failure of zVAD.fmk to inhibit cell death induced by doxorubicin or vincristine [23]. The novel observation that zVAD.fmk alters the baseline of ΔΨm also raises further questions about the function of caspases on this intracellular organelle.

Although the cellular response to TS inhibition was examined after 12 and 24 h exposures to Thymitaq, we were unable to determine the precise sequence of these cell cycle and mitochondrial events. Low levels of TS inhibition did not induce any change within 12 h, but led to S-phase arrest, DNA damage and mitochondrial changes evident by 24 h. In response to higher concentrations, S-phase arrest was observed and apoptosis was directly induced. Whether the mitochondrial alterations preceded or were concomitant with endonuclease-

Table 3 Cell cycle distribution and mitochondrial membrane potential ($\Delta\Psi$m) of leukaemia cells 24 h after continuous exposure to Thymitaq and/or zVAD.fmk. Values are the means ± SEM from three or four independent experiments

Cell line	Thymitaq (μM)	zVAD.fmk (μM)	Cell cycle distribution				$\Delta\Psi$m		
			Sub-G_1	G_1	S	G_2 ± M	Low	Normal	High
L1210	0		2 ± 0.8	44 ± 4.5	28 ± 2.9	26 ± 1.9	2 ± 0.4	96 ± 0.9	2 ± 0.6
	5		8 ± 1.7	15 ± 3.6	59 ± 3.4	21 ± 0.5	7 ± 3.0	69 ± 6.9	28 ± 7.6
	50		23 ± 4.9	45 ± 7	22 ± 1.1	15 ± 1.9	8 ± 2.3	82 ± 6	10 ± 4
	0	50	3 ± 0.8	52 ± 1.4	23 ± 2.3	23 ± 1.3	9 ± 3.0*	88 ± 2.5*	2 ± 0.9
	5	50	7 ± 1.4	24 ± 6.0	50 ± 5.9	20 ± 0.7	7 ± 3.0	79 ± 2.5	14 ± 4.5
	50	50	7 ± 0.9*	48 ± 1.8	27 ± 0.5	17 ± 0.6	8 ± 3.2	81 ± 7	10 ± 6.3
HL60	0		1 ± 0.1	50 ± 0.9	24 ± 1.3	27 ± 1.5	3 ± 0.9	95 ± 0.8	1 ± 0.3
	5		15 ± 5.0	24 ± 4.0	47 ± 3.5	17 ± 2.4	12 ± 3.7	76 ± 4.5	12 ± 1.1
	50		38 ± 3.6	44 ± 2.2	11 ± 1.4	7 ± 1.4	21 ± 1.8	74 ± 1.5	5 ± 1.7
	0	50	6 ± 0.9**	56 ± 2.0*	19 ± 1.2*	21 ± 2.2*	10 ± 2.1**	88 ± 1.8**	1 ± 0.2
	5	50	20 ± 5.0	36 ± 2.3*	36 ± 3.9	11 ± 1.3	19 ± 6.8	76 ± 6.3	5 ± 1.9*
	50	50	50 ± 4.3*	35 ± 2.9*	10 ± 1.9	6 ± 1.3	29 ± 10	67 ± 8.5	4 ± 1.2

* $P < 0.05$, ** $P < 0.01$, vs the corresponding dose of Thymitaq alone

induced DNA degradation could not be determined. Studies by others suggest that a reduction in $\Delta\Psi$m occurs first and induces the release of an apoptotic factor that stimulates the degradation of nuclear DNA [12, 22]. Analysis of our data showed that alterations in $\Delta\Psi$m correlated strongly with DNA degradation (sub-G_1), but a cause-and-effect relationship between these parameters could not be established.

A new finding was that a hyperpolarized $\Delta\Psi$m correlated with S-phase arrest, but again a cause-and-effect relationship was not established. Furthermore, S-phase arrest does not appear to be a necessary event for the induction of mitochondrial changes or apoptotic cell death. A causal link seems unlikely since our studies showed that high concentrations of Thymitaq failed to induce S-phase arrest but were still able to produce apoptosis, and others have shown that agents which do not mediate their effects via S-phase arrest still induce mitochondrial changes and apoptotic cell death [22, 30]. The relationship between DNA damage, mitochondrial membrane changes and caspase activation is unclear and needs further study.

In conclusion, TS inhibition induced by Thymitaq in L1210 and HL60 leukaemia cells resulted in apoptotic cell death, characterized by the presence of membrane blebbing, DNA fragmentation, apoptotic bodies, and biphasic alterations in $\Delta\Psi$m. This response was dependent on the duration and concentration of Thymitaq treatment, with low concentrations of Thymitaq inducing S-phase arrest and apoptotic cell death, and higher concentrations directly inducing apoptotic cell death. Caspase activation occurred downstream of the mitochondria in L1210 cells. In contrast, inhibition of caspase activation in HL60 cells increased the death response to TS inhibition by mechanisms unknown. The downstream biochemical pathway between TS inhibition and the subsequent death of cells needs further study, but caspase activation and the mitochondria have central roles in this response.

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