

Preferential induction of apoptosis of leukaemic cells by farnesol

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Abstract Farnesol preferentially inhibits proliferation and induces apoptosis of tumour-derived but not non-transformed cell lines. We investigated whether farnesol induces apoptosis of blasts from patients with acute myeloid leukaemia (AML) and leukaemic cell lines, as compared with normal, human primary haemopoietic cells. We show that 30 μ M farnesol causes apoptosis of leukaemic cell lines of T- and B-lymphocyte, myeloid or erythroid lineages and primary blasts obtained from patients with AML. However, the same concentration did not kill primary monocytes, or quiescent or proliferating T-lymphocytes. We conclude that farnesol selectively kills AML blasts and leukaemic cell lines in preference to primary haemopoietic cells.

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Key words: Farnesol; Apoptosis; Cell cycle; Leukemia

1. Introduction

Farnesol is a member of a class of compounds known as non-sterol isoprenoids [1]. Sterol and non-sterol isoprenoids are natural products which are produced from a common precursor, mevalonate, and are required for a number of cellular functions, such as cell signalling, protein synthesis, membrane integrity, cell proliferation and apoptosis [2]. These diverse cellular functions are mediated via the production from mevalonate of a number of compounds including cholesterol, ubiquinone, farnesyl and geranylgeranyl isoprenoids, dolichol and retinoic acid precursors [2,3]. Farnesyl diphosphate and geranylgeranyl diphosphate are necessary for protein prenylation, which is a post-translational modification of proteins such as Ras and which is required for their targeting to the plasma membrane and hence activity [4].

Cancer chemoprevention using monoterpenes is an active area [5]. The oily extracts from natural sources such as oranges, lemons and lemon grass contain a number of terpenes that possess anti-carcinogenic activity, including limonene, menthol and carvone. However, terpenoid compounds that possess hydroxy groups are generally more active than the corresponding terpene hydrocarbons. Examples include the inhibition of human MIA PaCa2 pancreatic tumour cells by

the terpenoid alcohols perillyl alcohol, geraniol and farnesol at respective in vivo concentrations of 300, 100 and 25 μ M [6].

Farnesol and geranylgeraniol have been shown to arrest the proliferation of a number of cell lines [6,7] and to induce apoptosis of several tumour-derived cell lines [8–10]. It was also reported that farnesol preferentially caused apoptosis of tumour-derived cell lines rather than in lines derived from non-neoplastic cells [10]. In a recent study [11], it was shown that farnesol and geranylgeraniol induced G0/G1 cell cycle arrest and apoptosis of a human lung adenocarcinoma cell line independent of protein prenylation. Several studies have shown that these compounds inhibit choline phosphotransferase and thereby prevent phosphatidyl choline biosynthesis [9,12–14].

Another likely mode of action of such terpenoids is by inhibition of protein prenyltransferases [15]. Inhibition of farnesyltransferase (Ftase) has been shown to cause tumour regression in animal models. Such enzyme inhibition was strongly reinforced by the recent report of the crystal structure of protein Ftase [16]. Ftase catalyses the lipidation of Ras at the carboxy terminus and other cellular signal transduction proteins. The size, shape and constitution of one cleft of Ftase is highly suited to the docking of a farnesyl isoprenoid. Farnesol induces apoptosis in lymphoma, cervical carcinoma and other cell types, and its relatively high anticarcinogen activity amongst simple terpenoid alcohols led us to study its effects on leukaemic cells.

Farnesol inhibits cell proliferation and induces apoptosis preferentially of neoplastic cells, albeit of cell lines. Therefore, we investigated whether farnesol causes cell cycle inhibition and apoptosis of human primary haemopoietic cells as compared with leukaemic cell lines and blasts from patients with myeloid leukaemia (AML).

2. Materials and methods

2.1. Reagents

Chemicals were obtained from Sigma unless stated otherwise. Farnesol (*trans*, *trans*-farnesol ([2E, 6E]-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol)) and taxol were diluted in dimethyl sulfoxide (DMSO) prior to use.

2.2. Cells and cell culture

2.2.1. Normal, primary haemopoietic cells. All cells were isolated from the blood of haematologically normal, healthy volunteers. Cell isolation and culture methods have been described previously [17,18] with additional details as described below. Briefly, mononuclear cells were obtained by Ficoll–Hypaque gradient centrifugation and monocytes were either obtained by adhesion to plastic or by selection with anti CD14-conjugated magnetic beads, according to the manufacturer's instructions (Miltenyi). Primary T-lymphocytes were obtained after the monocytes had been removed (mixture of T- and B-lymphocytes) and were at least 80% pure, as determined by flow cytometry with CD3, CD4, CD8, CD14, CD19 and CD45. The purified T-cells,

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which are in G0 [19], were cultured at 1×10^6 cells/ml in RPMI/10% FCS (Gibco-BRL)/penicillin/streptomycin. Where indicated, they were stimulated to enter G1 with 1 μ g/ml phytohaemagglutinin (PHA) (Glaxo-Wellcome) for 60 h and proliferating cells were obtained by adding 20 ng/ml interleukin-2 (IL-2) (Murex) for 5–7 days. During this period cells were kept between $0.8\text{--}2 \times 10^6$ /ml by adding fresh medium containing IL-2.

2.2.2. Leukaemic blasts. Blood was obtained from patients with acute myeloid leukaemia before chemotherapy. All the samples used had a high blast count ($>70\%$; as determined by staining with May Grunwald Giemsa (MGG) stain) and the mononuclear cells were purified by Ficoll–Hypaque gradient centrifugation. These were cultured in RPMI-1640/10% (v/v) FCS/penicillin/streptomycin containing 50 ng/ml each of GM-CSF, IL-3 and SCF.

2.2.3. Cell lines. Daudi (obtained from Dr Ian Kerr, London), Jurkat and HL-60 cells (from Dr Pamela Roberts, London) were maintained between $2\text{--}10 \times 10^5$ cells/ml in RPMI/10% (v/v) FCS and were split to $2\text{--}4 \times 10^5$ cells/ml 24 h before each experiment. TF-1 cells (from Dr Kitamura, Tokyo) were maintained between $5\text{--}50 \times 10^4$ /ml in RPMI/10% (v/v) FCS containing 10 ng/ml GM-CSF.

2.3. Counting viable cells

The proportion of viable cells which exclude trypan blue and dead cells staining blue was determined by counting at least 100 cells for each sample with an improved Neubauer chamber.

2.4. Flow cytometry

Cells in each cell cycle phase were quantified by two-colour flow cytometry of DNA (stained with propidium iodide (PI)) and total cell protein (stained with fluorescein isothiocyanate (FITC)) [20,21]. The proportion of cells undergoing apoptosis with sub-G1 DNA and low protein staining [22] was also determined. Analysis was carried out using an Epics-Elite flow cytometer (Coulter Electronics). Doublets of cells in G1 can contaminate the G2/M fraction and doublets of apoptotic cells can contaminate the S-phase fraction. Doublets were excluded as far as possible by only including cells which were within linear gates for forward scatter vs forward scatter peak, for FITC staining vs FITC peak and also for PI staining vs PI peak.

2.5. Staining slides

Slides of each cell sample ($0.5\text{--}1 \times 10^5$ cells) were prepared by cytocentrifugation (Shandon cytocentrifuge 3) for 7 min at 700 rpm. These were left to dry at room temperature for 24 h and were then stored at -20°C . After thawing, each slide was stained with MGG stain.

3. Results

3.1. Induction of apoptosis of leukaemic cell lines

In order to ascertain the concentration of farnesol which causes cell cycle arrest and apoptosis we cultured the following leukaemic cell lines: Jurkat (acute T-cell leukaemia), Daudi (B-lymphocyte from a patient with Burkitt's lymphoma), HL-60 (acute pro-myelocytic leukaemia) and TF-1 (erythro-leukaemic cell line derived from bone marrow) in the presence of $30\text{--}75 \mu\text{M}$ farnesol or diluent controls. After 24 and 48 h we determined the number of live and dead cells by manual counting. Also, samples were stained for flow cytometric anal-

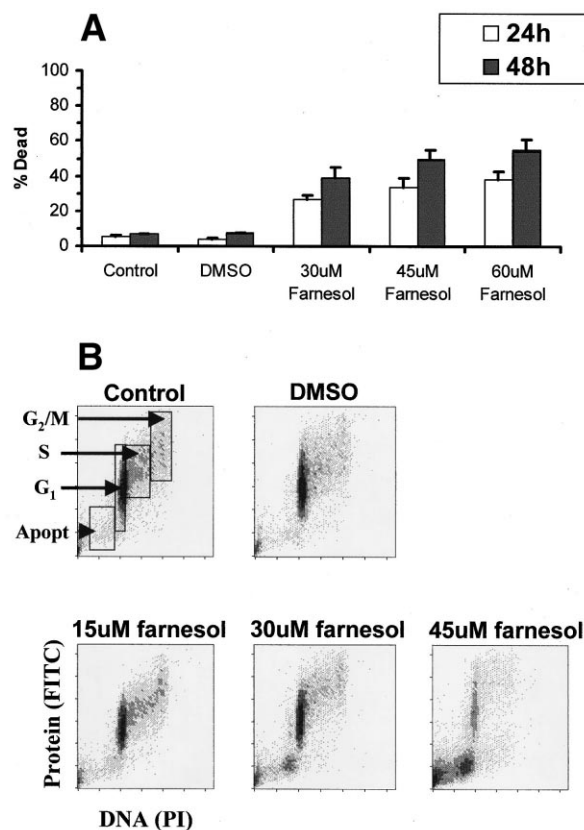


Fig. 1. A: Farnesol causes cell death of Jurkat T-cells. Jurkat cells were cultured in the presence of 30, 45 or 60 μM farnesol, with DMSO (equivalent to that added in the 60 μM farnesol sample) or with no addition, samples were taken after 24 and 48 h and viable cells which exclude trypan blue and dead cells which take up trypan blue were counted manually. The percentage of dead cells for each condition is shown (mean $\pm$ S.E.M., $n=3$). B: Flow cytometric analysis of Jurkat cells. Jurkat cells were cultured for 48 h with: a. no addition, b. DMSO, c. 15 μM , d. 30 μM or e. 45 μM farnesol and samples were analysed for DNA and total protein content as described in Section 2. Cells in the G1, S and G2/M phases of the cell cycle are indicated as well as those undergoing apoptosis which have sub-G1 DNA staining and low protein content.

ysis of DNA and protein content in order to determine the percentage in different cell cycle phases as well as those which had died by apoptosis. A significant number of dead cells were detected at 24 and 48 h in cultures of Jurkat cells containing greater than 15 μM farnesol, as judged by trypan blue staining, and this was increased at higher concentrations of the drug (Fig. 1A). Farnesol caused an increase in cells with a sub-diploid DNA and low protein content characteristic of cells undergoing apoptosis. An example of such an experiment

Table 1
The effect of farnesol on the percentage of Jurkat cells in each cell cycle phase ($n=4$)

		Control	Control+ DMSO	Farnesol (15 μM)	Farnesol (30 μM)	Farnesol (45 μM)	Farnesol (60 μM)	Farnesol (75 μM)
24 h	Sub G1	5.6	4.5	7.4	26.3	37.5	41.8	41.0
	G1	50.6	51.0	49.0	41.9	33.6	23.5	25.0
	S	31.3	31.7	33.2	26.3	23.7	25.5	24.7
	G2+M	31.3	13.1	10.9	8.8	5.9	8.9	9.2
48 h	Sub G1	6.9	7.7	9.5	29.5	53.1	63.7	70.8
	G1	52.4	52.3	54.5	41.8	24.4	19.5	15.4
	S	30.5	29.2	27.0	21.4	16.8	14.0	11.5
	G2+M	11.0	10.9	9.1	7.3	4.5	3.2	2.6

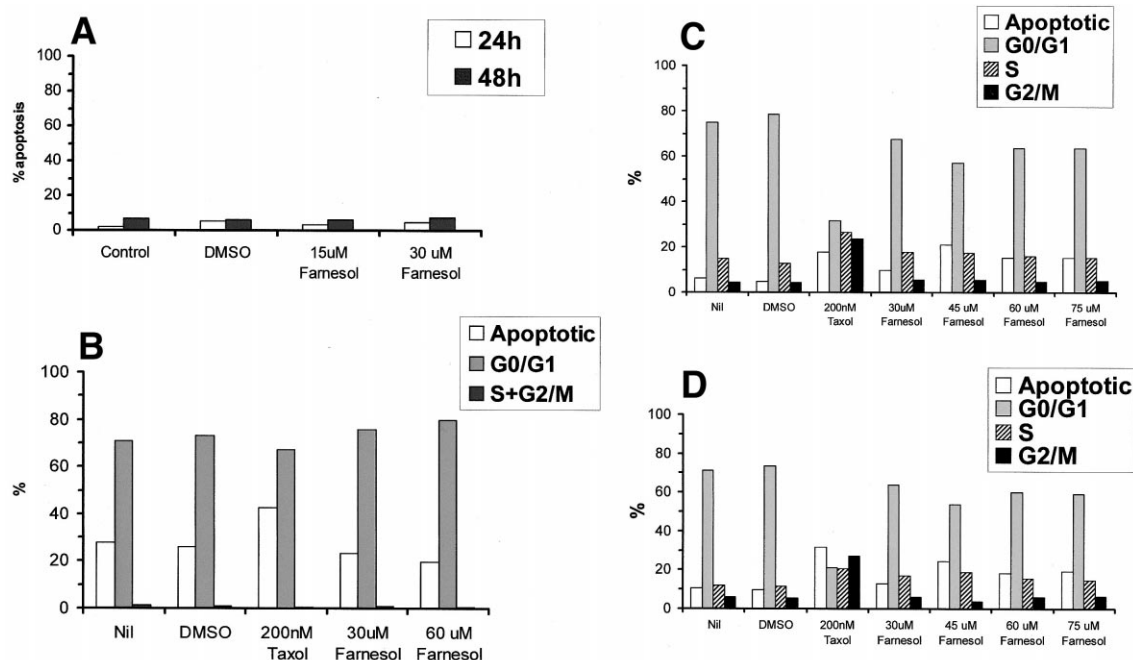


Fig. 2. A: Farnesol does not cause apoptosis of primary T-lymphocytes. Primary T-lymphocytes isolated from peripheral blood were cultured under the conditions shown for 24 and 48 h and samples were analysed as in Fig. 1B. The percentage of apoptotic cells with sub-G1 DNA staining and low protein content is shown. B: Farnesol does not cause apoptosis of primary monocytes. Monocytes were isolated from peripheral blood, cultured under the conditions indicated for 48 h and analysed as in Fig. 1B. The percentages in G0/G1, S+G2/M or undergoing apoptosis are shown (representative of $n=2$). C and D: Farnesol does not cause apoptosis of proliferating primary T-cells. Peripheral blood T-cells were cultured in PHA and then stimulated to proliferate with IL-2 as described in Section 2. Samples were taken at 24 h (C) and 48 h (D) and analysed as described for Fig. 1B (representative of $n=3$).

is shown in Fig. 1B and the data from four experiments are tabulated (Table 1). Data for TF-1 cells at 45 μ M farnesol are shown in Table 2 and similar data showing significant cell death were obtained for both HL-60 and Daudi cells (data not shown). Our results are within the concentration range reported by others for inducing apoptosis of the CEM-1 acute leukaemia T-cell line [8].

3.2. Farnesol does not cause apoptosis of primary lymphocytes or monocytes

Next we determined whether the same concentrations of farnesol induced apoptosis of purified monocytes or T-lymphocytes isolated from peripheral blood. Farnesol at 15 or 30 μ M caused little death of quiescent, primary T-lymphocytes over a 48 h period, as judged either by trypan blue exclusion (not shown) or flow cytometric analysis of DNA content ($<7\%$ dead; Fig. 2A). In order to determine whether another primary haemopoietic cell of a different lineage was killed by farnesol we isolated monocytes from peripheral blood and cultured them in up to 60 μ M farnesol for 48 h. We did not detect apoptosis above control values, as judged by flow cytometry of DNA content (Fig. 2B). Thus significantly fewer primary T-lymphocytes or monocytes die in response to farnesol than the any of the leukaemic cell lines tested.

Both T-lymphocytes and monocytes are quiescent and the difference in the effects of farnesol between these and leukaemic cell lines could occur if farnesol preferentially kills proliferating cells. In order to address this possibility, primary T-lymphocytes proliferating in IL-2 were cultured with 30–75 μ M farnesol for 24 and 48 h and the same assays were performed. As shown in Fig. 2C,D, farnesol caused $<10\%$

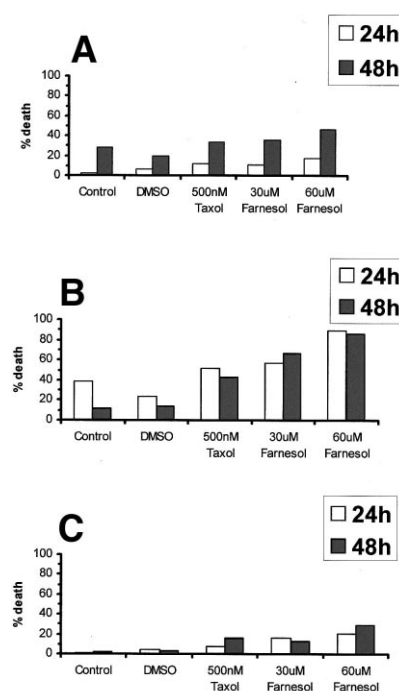


Fig. 3. A–C: Farnesol causes cell death of blasts isolated from patients with AML. AML blasts were isolated as described in Section 2 and cultured with SCF, IL-3 and GM-CSF. Farnesol or DMSO were added as indicated and cells were counted manually after 24 and 48 h. The percentages of dead cells which took up trypan blue are shown for three patients.

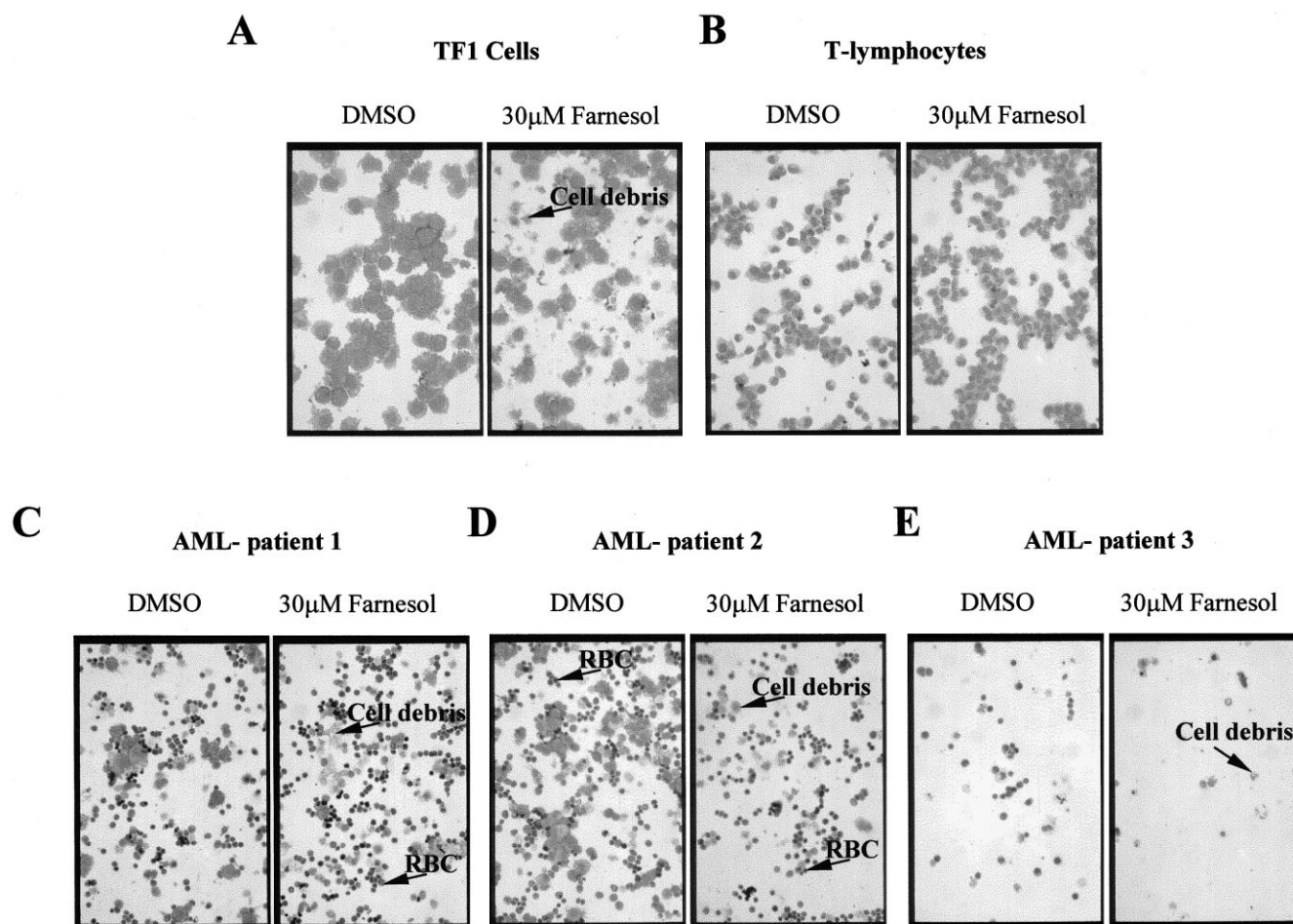


Fig. 4. A–E: Cell debris in TF1 and AML blasts treated with farnesol. A: TF1 cells. B: T-lymphocytes proliferating in IL-2. C–E: Three AML samples were cultured with diluent (DMSO) or with 30 μ M farnesol for 72 h (D for 48 h) and cytocentrifuge preparations were stained with MGG. Cell debris and contaminating red blood cells (RBC) are shown.

apoptosis above the controls and the percentage undergoing apoptosis did not increase at 72 h (not shown). We also cultured the same cells with taxol which caused cell cycle arrest in G2/M and did induce apoptosis of these primary cells. Thus, farnesol selectively kills leukaemic cell lines and does not induce apoptosis simply because cells are proliferating.

3.3. Farnesol reduces the number of viable AML blasts

In order to determine whether farnesol would kill primary leukaemic cells we cultured blasts isolated from three patients with AML with 30 and 60 μ M farnesol and counted viable

Table 2

The effect of farnesol on the percentage of TF1 cells in each cell cycle phase ($n = 4$)

		Control	Control+ DMSO	Farnesol (45 μ M)
24 h	Sub G1	32.0	27.2	68.2
	G1	42.8	44.4	23.2
	S	20.6	22.6	6.0
	G2+M	5.2	6.5	2.4
48 h	Sub G1	19.1	39.8	90.1
	G1	54.8	43.8	8.2
	S	18.8	11.9	1.2
	G2+M	7.3	4.6	0.2

cells after 24 and 48 h. In each case, both concentrations of farnesol caused significant cell death of cells from each of the patients analysed, as compared with the diluent controls (Fig. 3A–C). We note, however, that the proportion of dead cells detected varied between 25 and 90% for the samples tested. Staining cytocentrifuge preparations showed that there were significant numbers of cell debris in the farnesol-treated samples as compared with the diluent controls (Fig. 4C–E). Slides of TF1 cells and proliferating primary T-lymphocytes cultured with farnesol were also stained and cell debris was present in the sample of farnesol-treated TF1 cells (Fig. 4A) but not in farnesol-treated T-lymphocytes (Fig. 4B). We concluded that farnesol induces apoptosis of blasts isolated from AML patients.

4. Discussion

In this study we have investigated whether farnesol selectively kills leukaemic cells as compared with normal haemopoietic controls. To this end we determined the concentration of farnesol which causes the apoptosis of a panel of leukaemic cell lines corresponding to haemopoietic cells of different lineages. We then showed that the same concentration does not cause the death of normal, primary T-lymphocytes or mono-

cytes. Finally, we have shown that farnesol causes cell death of blasts obtained from patients with AML. Our data show that farnesol selectively kills primary leukaemic cells and cell lines rather than normal primary haemopoietic cells.

Previous work showed that farnesol causes apoptosis of a range of different malignant cell lines at concentrations between 15 and 30 μ M [10,13–15]. Our data are consistent with this and there was significant cell death after 24 h of all the leukaemic cell lines tested at these concentrations. When cells die by apoptosis they lose cytosol by blebbing and so become smaller and their DNA becomes fragmented. Thus our two-colour flow cytometric data also indicate that apoptosis occurred principally from the G0/G1 phase: we observed cells with sub-G1 DNA and low protein content but there was no evidence of cells with sub-G2/M or sub-S-phase DNA and low protein content. These data are in agreement with the effects of farnesol on the human adenocarcinoma cell line, A549 [11].

Farnesol does not induce apoptosis of human primary T-lymphocytes or monocytes isolated from peripheral blood. We increased the concentration of farnesol in cultures of quiescent T-lymphocytes and even 90 μ M farnesol did not cause apoptosis over the period of study (data not shown). It is clear also that the proliferation state of the primary cells is not important since farnesol did not cause apoptosis of either non-proliferating or proliferating primary T-lymphocytes. However, it is not clear why farnesol does not kill primary haemopoietic cells. Others have shown that the effects of farnesol on cell lines are mediated through the inhibition of choline phosphotransferase rather than blocking isoprenoid biosynthesis from mevalonate [10] and can be reversed by the addition of diacylglycerol or phosphatidyl choline [11,14]. Further work is required to determine whether farnesol inhibits choline phosphotransferase in normal, primary haemopoietic cells and if so what downstream mechanisms protect these cells, and why leukaemic cells are susceptible.

Our data show that farnesol does cause primary AML blasts to die over a period of 24 h. There was variation on the percentage of cell death between samples from the three patients, but each was significantly above the diluent controls. It is possible that the variation is due to a difference in leukaemic cell type and more extensive studies are now warranted to test whether AML cells of different FAB-types are more or less susceptible to farnesol. In conclusion, our studies show that farnesol causes significant cell death of leukaemic cells without affecting normal, primary cells.

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