Alpha Terpineol: A Potential Anticancer Agent which Acts through Suppressing NF-κB Signalling

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Abstract. *Background: Alpha terpineol is a bioactive component of Salvia libanotica essential oil extract and has shown antitumour activity. Materials and Methods: The cytotoxicity of alpha terpineol towards different tumour cell lines was evaluated in vitro. Mechanistic characterization was performed using analysis of drug activity in a cell line panel and drug-induced gene expression perturbation using the connectivity map approach. Results: The small cell lung carcinoma was the cell line most sensitive to alpha terpineol. The results proposed alpha terpineol as an NF-κB inhibitor, which was confirmed by the observed dose-dependent inhibition of NF-κB translocation and activity using two NF-κB assays, and by the down-regulation of the expression of several NF-κB-related genes such as IL-1β and IL1R1. Conclusion: The results suggest that alpha terpineol inhibits the growth of tumour cells through a mechanism that involves inhibition of the NF-κB pathway.*

Salvia libanotica (sage) is a species endemic to the Eastern Mediterranean. Its essential oil extract has been found to suppress the formation of skin papillomas, to exert anti-inflammatory effects on the skin of treated mice (1) and to have strong antimicrobial properties (2). It has been demonstrated that the three bioactive components, namely alpha terpineol, linalyl acetate and camphor, synergise to induce cell cycle arrest and apoptosis, mainly *via* mitochondrial damage (cytochrome *c* release), caspase activation, and PARP cleavage, in human colorectal cancer cells (3). Recently, it was shown that linalyl acetate has cytotoxic effects on several types of human tumour cells; small cell lung carcinoma and colorectal cancer cell lines were the most sensitive (4). It was proposed that protein kinases and/or the nuclear factor κB (NF-κB) pathway are possible targets for linalyl acetate cytotoxicity (4).

Alpha terpineol, a volatile monoterpenoid alcohol, is a major component of the essential oil of many plants and has been reported to enhance the permeability of skin to lipid-soluble compounds (5). Previous reports showed that alpha terpineol exhibits anti-proliferative effects on human erythroleukaemic cells (6). Alpha terpineol has also been described to have anti-inflammatory properties (7), as it was found to be a potent inhibitor of superoxide production, selectively regulating cell function during inflammation (8). Alpha terpineol has been also shown to have antibacterial (9) and antifungal activities (10).

Activation of the transcription factor NF-κB is one reason for the established link between chronic inflammation and the development of cancers and is widely known to induce the expression of diverse target genes involved in immune responses, inflammation, cell survival and cancer. Many different types of human tumours express NF-κB constitutively. It has been suggested that blocking NF-κB can cause tumour cells to stop proliferating, die, or become more sensitive to the action of anti-tumour agents (11).

In the present study, several approaches were used to characterize the anti-tumour activity and to elucidate the mechanism of action of alpha terpineol: drug-response analysis in a panel of cell lines, as well as in primary human tumour and normal mononuclear cells; cell cycle analysis, micro-array gene expression analysis; and the gene expression signature-based screening approach, the connectivity map. Based on the results of these approaches, alpha terpineol appears to be an NF-κB/ protein kinase inhibitor. The NF-κB/protein kinase inhibitory hypothesis was validated by performing preliminary mechanistic studies which supported the hypothesis.

Materials and Methods

Cell line, primary human tumour and normal mononuclear cultures. The panel of the ten human cancer cell lines that was used has been described previously (12). The panel consists of the parental cell lines RPMI 8226 (myeloma), CCRF-CEM (leukaemia), U937-GTB (lymphoma) and NCI-H69 (small cell lung cancer); the drug-

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resistant sublines 8226/Dox40, 8226/LR5, CEMVM-1, U937-vcr, H69AR and the primary resistant ACHN (renal adenocarcinoma). All cells were grown in RPMI-1640 culture medium. Four colorectal cell lines, HCT-8, SW620, HCT-116 and HT-29 obtained from ATCC (Manassas, VA, USA) were cultured in RPMI-1640, Leibovitz's L-15, McCoy's and Dulbecco's modified Eagle's media (DMEM) respectively. The breast cancer epithelial cell line, MCF7 and the human cervix (Hela) cell lines obtained from ATCC were cultured in DMEM deprived of phenol red and Eagle's Minimal Essential medium with 1 mM sodium pyruvate, respectively. All cell lines were supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 μg/ml streptomycin and 100 IE/ml penicillin (all from Sigma Aldrich Co., St Louis, MO, USA) and incubated at 37˚C in humidified air containing 5% CO_2. The resistant cell lines were tested regularly for maintained resistance to the selected drugs. Growth and morphology of all cell lines were monitored on a weekly basis.

Tumour cell samples were obtained from two patients with chronic lymphocytic leukaemia (CLL). The patient samples were obtained from peripheral blood. Leukaemia cells were isolated from the blood by density gradient centrifugation on a 1.077 g/ml Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient. CLL patients were previously treated with standard cytotoxic drugs. Cell viability was determined by trypan blue exclusion test and the proportion of tumour cells in the preparation was judged by inspection of May-Grunwald-Giemsa-stained cytospin preparations by a haematologist. RPMI-1640 cell culture medium (supplemented as described above) was used throughout. Normal mononuclear cells (PBMCs) from two healthy donors were collected and prepared in the same way as leukaemia samples.

Drug exposure and viability assays. Alpha terpineol (Across, Belgium) was tested in duplicate at 8 concentrations using 2-fold serial dilutions from a maximum of 50 mM. Alpha terpineol was supplied in liquid form and was further diluted in sterile water. 384-Well microtitre plates (Nunc, Roskilde, Denmark) were prepared with 5 μl drug solution in 10 times the final drug concentration using a BioMek 2000 pipetting robot (Beckman Coulter, Fullerton, CA, USA).

Tumour cells of the cell line panel, colorectal cell lines (5000 cells per well), CLL cells and PBMCs (50000 cells per well) were seeded separately in the drug-prepared 384-well plates using a Precision 2000 pipetting robot (Bio-Tek Instruments Inc., Winooski, VT, USA). Three columns without drugs served as controls and one column with medium only served as a blank. The plates were incubated at 37˚C for 72 h and were then analysed using a fluorometric microculture cytotoxicity assay (FMCA).

The FMCA, described in detail previously (13, 14), was performed to measure cell survival, based on the measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes. Briefly, the cells were washed twice with PBS, 1 μl of 0.5 mg/ml FDA was added and after 50-70 min of incubation, the fluorescence, which is proportional to the number of living cells, was measured. Cell survival (survival index, SI) was defined as fluorescence in test wells in comparison to control wells, with blank values subtracted. Quality criteria for a successful assay included a mean coefficient of variation of less than 30% in the control and a fluorescence signal in control wells of more than five times the blank. Three repeat experiments were carried out where cell preparation, drug preparation as well as FMCA analysis were performed independently. Camphor was tested with all cell types except for the colorectal cancer lines.

Data from all three experiments were processed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) with non-linear regression to a standard sigmoidal dose-response model. 0% was set as baseline, 100% cell survival was set as the maximum effect and the inhibitory concentration 50% (IC_{50}) was estimated. When the exposure to the test substances did not result in 50% reduction in cell survival in the concentration range tested, the IC_{50} was set to the highest concentration used, and when the exposure to the test substances resulted in less than 50% reduction in cell survival in the concentration range tested, it was set to the lowest concentration used.

Cell cycle analysis. The lymphoma cells, U937-GTB, were seeded in 12-well plates at 1×10^6 cells/ml and treated with 3 or 6 mM alpha terpineol for 6 h or 24 h. At the end of the exposure time, cells were collected, rinsed twice with PBS and fixed in 70% ice-cold ethanol at 2×10^5 and stored at 0-4˚C until analysis. The fixed cells were then centrifuged, the cell pellet was rinsed with PBS and then suspended after centrifugation in 1 ml of 0.02 mg/ml propidium iodide staining solution with 0.2 mg/ml DNase-free RNase and kept for ≥2 h. DNA staining was then detected using an LSR II flow cytometer. ModFit LT (Verity Software House, Topsham, USA) software was used to analyse the percentage of the cells in different cell cycle phases.

Correlation to drugs in database. A total of 400 investigational substances and standard drugs with a known mechanism of action representing different mechanistic groups were tested in the 10 tumour cell line panel using the 72 h FMCA, and their IC_{50} were estimated and included in the database. The estimated IC_{50s} of alpha terpineol in the cell line panel were used in the database and a correlation analysis was performed. A correlation coefficient ≥0.7 was used to identify the drugs that correlated with alpha terpineol activity. Resistance factors were defined as IC_{50} values in the resistant subline divided by IC_{50} values in the parental cell line.

Microarray gene expression analysis. MCF7 cells were treated with PBS, 1.2 mM alpha terpineol or with vehicle for 6 h in RPMI-1640 with 10% FCS. RNA was isolated using Qiagen columns (Qiagen GmbH, Hilden, Germany). RNA concentration for the two samples was measured using an ND-1000 spectrophotometer (NanoDropTechnologies, Wilmington, DE, USA) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc., Palo Alto, CA, USA). Total RNA (2 μg) from each sample were used to prepare biotinylated fragmented cRNA according to the GeneChip® Expression Analysis Technical Manual (Rev. 5, Affymetrix Inc., Santa Clara, CA, USA). Affymetrix GeneChip® Expression Arrays (Human Genome U133 Plus 2.0 Array) were hybridised for 16 h in an incubator at 45˚C, rotated at 60 rpm. According to the GeneChip® Expression Analysis Technical Manual, the arrays were then washed and stained using a Fluidics Station 450 and scanned using an Affymetrix GeneChip® Scanner 3000 7G.

Connectivity Map data analysis. The raw data from the microarray expression analysis were normalised using the robust multi-array average (RMA) method (15). Genes were then ranked according to the expression differences compared to the vehicle-treated control, and the 100 genes that increased or decreased most compared to the

vehicle-treated control were used as 'signatures' in the next step of the analysis. To investigate the target activity of the examined compound, an approach was used relating the activity of alpha terpineol to drugs with known biological activities at the gene expression level (16). The current version of the Connectivity Map data set (www.broad.mit.edu/cmap) contains genome-wide expression data for 6100 treatment and vehicle control pairs, representing 1309 distinct small molecule bioactive compounds (perturbagens). Enrichment of both the up- and down-regulated genes from a given signature in the profiles of each treatment instance was estimated (16) and combined to produce a 'connectivity score'. Instances were then ranked in descending order of connectivity score. A 6 h treatment time was chosen to capture the primary and potentially mechanistic effects of the compounds. The alpha terpineol query signature was loaded into the database and the query for substances that induce the biological state represented by the alpha terpineol signature was executed. To develop hypotheses on the mechanism of action of alpha terpineol, the first 50 instances were scanned out of the 6100 instances of the Connectivity Map resources, at the top of the list.

NF-κB translocation assay. Hela cells (1500 cells/90 μl/well) were seeded in standard black 96-well clear-bottom microplates (Packard ViewPlate®, Packard, USA) and left to adhere overnight before the addition of 10 μl of test compounds. In a preliminary experiment, Hela cells were exposed either to 6.25 mM alpha terpineol or 2.5 μM Celastrol which was obtained from MicroSource Discovery (Gaylordsville, CT, USA). The plate was incubated for 2 h at 37˚C. In the second experiment, alpha terpineol was tested at 3 concentrations from a maximum of 25 mM using 2-fold serial dilutions. Rottlerin, MG-132 and 15-delta prostaglandin J2 were obtained from Sigma-Aldrich and tested at 100, 20 and 10 μM respectively. The plate was incubated for 4 h at 37˚C. All concentrations were tested in triplicate. At the end of the incubation times, treated cells and positive control cells were stimulated with 50 ng/ml of tumour necrosis factor α (TNFα) for 30 min. Negative control cells were used as a reference to quantify the extent of baseline NF-κB translocation in the cell line studied.

NF-κB translocation into the nucleus was evaluated using the NF-κB Activation HCS HitKit™ (High Content Screening, obtained from Cellomics Inc., USA) utilising the ArrayScan® HCS reader (Cellomics Inc.) as described earlier (17). The ArrayScan® reader automatically quantifies the mean nuclear-cytoplasmic intensity difference of the amount of p65 immunofluorescence staining in a predefined number of cells (250 cells/well). The mean nuclear-cytoplasmic intensity difference was presented as % NF-κB translocation, defined as the mean nuclear-cytoplasmic intensity difference in the experimental wells as a percentage of that in control wells.

To determine the cytotoxicity of alpha terpineol in parallel to NF-κB translocation analysis, 96-well microtitre plates (Nunc) were prepared with 20 μl per well of drug solution at 10 times the desired concentration. Alpha terpineol was tested at 5 concentrations from a maximum of 12.5 mM using 2-fold serial dilutions. Each concentration was prepared in triplicate. Hela cells were seeded at 20,000 cells/180 μl and the plates were incubated at 37˚C for 72 h. At the end of the incubation period, the cell survival was determined with the FMCA as described above.

Transcriptional reporter gene assay for the NF-κB pathway. To further analyse the intracellular status of the NF-κB pathway upon exposure to alpha terpineol, GeneBLAzer® Technology was used (Invitrogen Corporation, Madison, Wisconsin, USA). The cervical human cell line, NF-κB-bla-ME 180-Inhibition Screen-Activated by TNF-α, which contains a beta-lactamase reporter gene under the control of the NF-κB response element stably integrated into ME-180 cells, was used for this purpose according to Invitrogen's standards. Alpha terpineol was tested at 5 concentrations from a maximum of 4.5 mM by 3-fold serial dilution. The IC_{50} was determined, in inhibitor (% inhibition) mode using the 10 point dose-response curves.

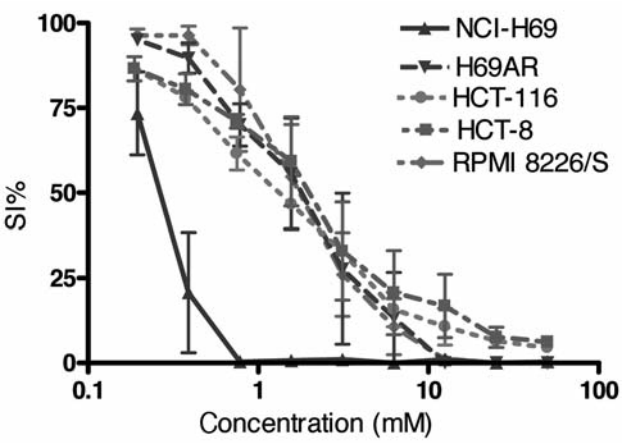

Figure 1. *Dose-response curves showing the effect of alpha terpineol in six of the 14 human tumour cell lines tested in this study as assayed by the FMCA. Data from 3 experiments are represented as mean±SEM.*

Kinase profiling. Alpha terpineol kinase profiling was performed against Invitrogen's Select Screen™ kinase panel of 50 key kinase targets (SelectScreen Kinome Sampler Panel) at a single concentration of 0.64 mM tested at 10 μM ATP. The kinases were selected to cover the kinome broadly and to provide a good approximation of compound potency. The assays were performed according to the Screening Protocol and Assay Conditions of Invitrogen. Dose-response curves, obtained from a maximum of 5.8 mM by 3-fold serial dilution, on JAK3, AKT1 and IKKbeta, were performed.

Results

The dose–response curves for alpha terpineol in 5 of the cell lines are shown in Figure 1. The small cell lung carcinoma cell line NCI-H69 was the most sensitive to alpha terpineol (IC_{50}: 0.26 mM). The colorectal cancer cell lines HCT-116 and HCT-8, the small cell lung carcinoma subline H69AR and the myeloma RPMI 8226/S cell lines were also sensitive to alpha terpineol. Alpha terpineol showed higher activity in tumour cells from CLL than in PBMCs (data not shown). The tumour selectivity ratio for alpha terpineol, defined as the ratio between the mean IC_{50} in PBMCs and CLL, was found to be around 3.

Overall, the resistance factors for alpha terpineol were low. The greatest difference in sensitivity between a parental line and its subline was observed for H69 and its subline H69AR, which was around 6. Resistance factors of around

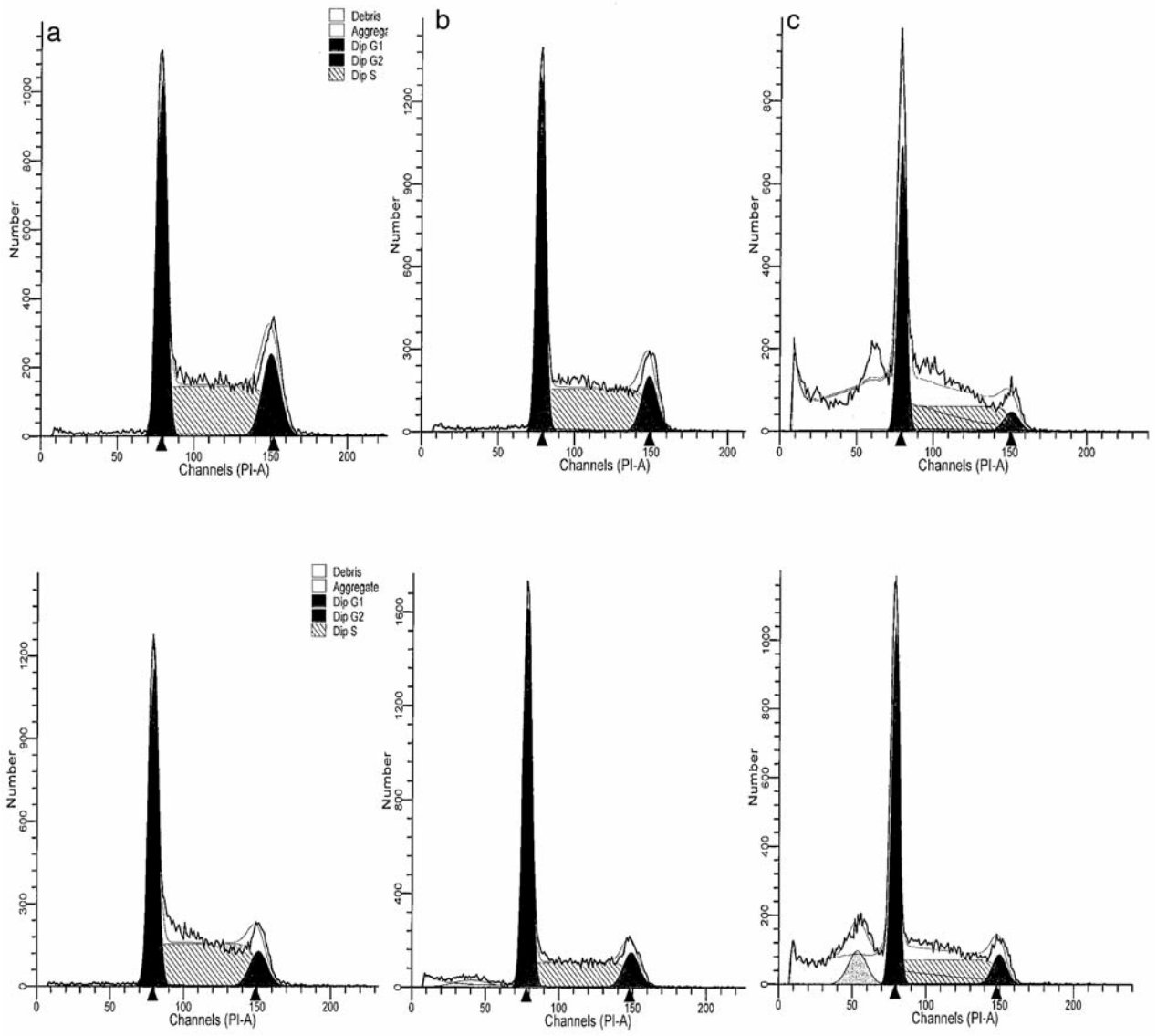

Figure 2. *Cell cycle regulation in the lymphoma cells, U937-GTB, induced by 3 mM (b and e) and 6 mM (c and f) alpha terpineol at 6h (b and c) and 24h (e and f) using the LSR II flow cytometer. ModFit LT software was used to analyse the percentage of the cells in different cell cycle phases. (a and d) are the control cell cycle profiles of U937-GTB lymphoma cells, at 6h and 24h, respectively.*

3 were observed for Pgp- and GSH-associated resistance (data not shown).

Flow cytometric analysis was used to examine the cell cycle changes induced by alpha terpineol in the lymphoma cells, U937-GTB. Alpha terpineol induced a concentration and time-dependent accumulation in G_0/G_1 (Figure 2). At 6 h and 24 h, 3 mM alpha terpineol induced an increase in G_0/G_1 up to 40% and 55% (Figures 2b and e) whereas 6 mM induced an increase in G_0/G_1 up to 49%, and 52% (Figures 2c and f) in comparison to 37% and 42% in the control cells (Figures 2a and d) at the 2 time points, respectively. This accumulation was accompanied with a parallel loss of cycling cells in S and G_2/M phases. The percentage of preG_1 (apoptotic) cells induced by 6 mM alpha terpineol at 6 and 24 h was 7.4% and 10.3%, respectively, as compared to only 0.04% and 0.23% in the control cells. Longer exposure time (24 h) was needed for 3 mM alpha terpineol to induce a slight change in the percentage of apoptotic cells (4.2%).

When comparing the log IC_{50} patterns in the drug activity database, the correlation coefficient of alpha terpineol was >0.8 with 6 of the 400 compounds included in the database, namely the sage oil component linalyl acetate, PKC412, thiordazine hydrochloride, pyrithione zinc, tyrphostin 25 and lanatoside C. The correlation coefficient was moderately

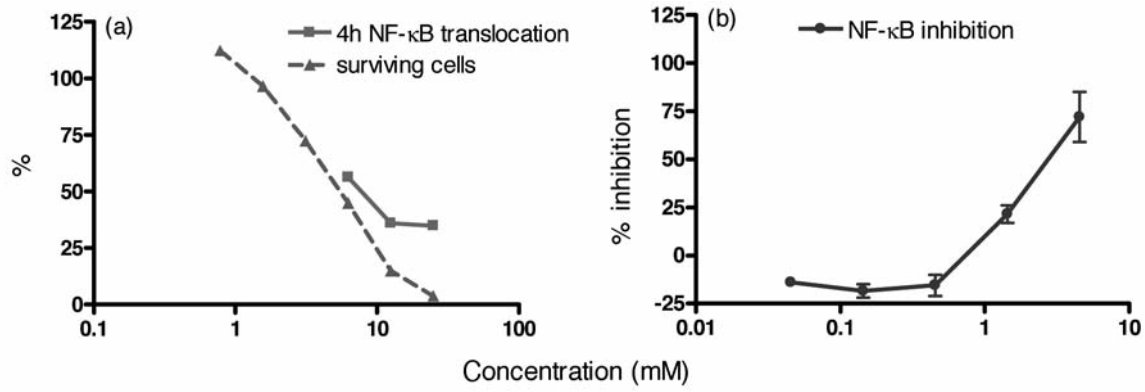

Figure 3. *Effect of different concentrations of alpha terpineol on: (a) TNFα-induced NF-κB translocation and percentage of surviving, human cervix (Hela) cells and (b) the intracellular status of NF-κB, activated with TNF-α, in the human cervical cell line, beta-lactamase reporter gene- ME-180. Data for cell survival are represented as the mean±SEM.*

Table I. *Correlation between the activity pattern of alpha terpineol, and drugs from the drug database with Pearson's correlation coefficient >0.7 and their proposed mechanism of action.*

Drug	Classification	Pearson's correlation coefficient
Linalyl acetate	NF-κB inhibitor (4)	0.94
PKC412	Akt kinase inhibitor (32)	0.86
Thiordazine hydrochloride	Antipsychotic, calmodulin inhibitor, anti-inflammatory, cyclooxygenase 2/lipoxygenase inhibitor.	0.85
Pyrithione zinc	NF-κB inhibitor (33)	0.85
Tyrphostin 25	EGF Tyrosine kinase, NF-κB inhibitor (34)	0.81
Lanatoside C	EGF Tyrosine kinase, NF-κB inhibitor (34)	0.80
Cetrimonium	Antifungal	0.79
Tricribine	PI3 kinase/AKT inhibitor (35)	0.78
Wortmannin	PI3 kinase/AKT inhibitor (36)	0.77
Thiothixene HCl	Antipsychotic thioxanthenes derivative	0.76
Acetochlor	Herbicide	0.76
Tyrphostin 47	Tyrosine kinase, NF-κB inhibitor (37)	0.74
Disulfiram	NF-κB, proteasome inhibitor (38)	0.73
Lavendustin A	Tyrosine kinase, NF-κB inhibitor (39)	0.71

high (0.79-0.71) with tricribine, wortmannin, tyrphostin 47, disulfiram and lavendustin A. A high correlation may suggest a common mechanism of action. The proposed mechanisms of action of these substances are presented in Table I, which shows that protein kinase and/or NF-κB inhibition is the common mechanism of action of these substances. Alpha terpineol activity was also correlated to compounds whose mechanism of action is not clearly known (Table I).

The up- and down-regulated genes obtained from the Affymetrix expression analysis of alpha terpineol effect on the breast cancer cell line MCF7, showed an association to several biological processes involving apoptosis and cell cycle. The Connectivity Map database identified several drugs having high similar gene expression profiles and inducing the biological state represented by the alpha terpineol signature (alpha terpineol query signature is provided in Online Resource 1). The scanning of the first 50 out of the 6100 instances of the Connectivity Map resources, at the top of the list, revealed that these 50 instances consisted of 36 different compounds. High connectivity scores were observed for multiple and single instances of 21 perturbagens in which NF-κB/protein kinase and calmodulin inhibition contributes to their anti-tumour activity (Table II). Four substances, thapsigargin, benzethonium chloride, methylbenzethonium chloride and chlorhexidine with multiple and single instances out of the remaining 15 compounds have previously been described in literature to induce loss in mitochondrial membrane potential a release of cytochrome *c* in tumour cells (18-20). However, the other 11 compounds of the top-50 ranking list included substances

Table II. *The perturbagens that highly correlated with alpha terpineol which were among the 50 highest ranking perturbagens identified through the Connectivity Map in which NF-κB/ protein kinase and calmodulin inhibition contributed to their anti-tumour activity.*

Connectivity Map name (n)[1]	Maximum connectivity score[2]	Rank[3]	Description
Ivermectin (1)	1.000	1	NF-κB inhibitor (40)
Pyrvinium (4)	0.995	2, 3, 28, 35	Akt kinase inhibitor (41)
Rottlerin (2)	0.963	7, 48	NF-κB (42), IKK, and Ca/calmodulin-dependent kinase II inhibitor
Phenoxybenzamine (3)	0.957	10, 12, 26	Antipsychotic, calmodulin inhibitor
Pimozide (1)	0.955	11	Antipsychotic, calmodulin inhibitor
Prenylamine (1)	0.942	13	Calmodulin inhibitor
Gossypol (1)	0.936	16	NF-κB (43), Akt kinase inhibitor
Astemizole (2)	0.921	17, 29	H1 receptor antagonist, NF-κB inhibitor
Fluspirilene (1)	0.92	18	Antipsychotic, calmodulin inhibitor
Mometasone (1)	0.909	20	Antiinflamatory, NF-κB inhibitor (44)
Terfenadine (2)	0.907	21, 32	H1 receptor antagonist, NF-κB inhibitor
Dequalinum chloride (2)	0.875	27,38	Calmodulin inhibitor
Valinomycin (2)	0.87	30, 31	Akt (45), p38MAPK, mitochondrial inhibitor
Disulfiram (1)	0.857	37	NF-κB, proteasome inhibitor (38)
Clotrimazole (1)	0.853	39	Calmodulin inhibitor
Thioridazine (1)	0.85	40	Calmodulin inhibitor, anti-inflammatory
Naftifine (1)	0.841	44	Anti-inflammatory
Celastrol (1)	0.833	46	NF-κB, proteasome inhibitor (46)
Oxaprocin (1)	0.831	47	NF-κB inhibitor (47)
Clioquinol (1)	0.821	49	NF-κB, proteasome inhibitor (48)
MG-262 (1)	0.821	50	Proteasome, NF-κB inhibitor (49)

[1](n): Number of treatment instances among the 50 top perturbagens; [2]maximum connectivity score of the perturbagen instances; [3]rank according to Connectivity Map score.

known to exhibit different or unknown mechanisms of action (data not shown). Given these results, the 50 high ranking perturbagens were considered to be likely relevant to the hypothesis generation process.

To test the hypothesis that alpha terpineol was acting on the NF-κB and/or protein kinase pathways, NF-κB and protein kinase profiling assays were performed. The NF-κB translocation assay showed that exposure of Hela cells to alpha terpineol inhibited TNFα-induced NF-κB translocation into the nucleus in a time- and dose-dependent manner. The survival of Hela cells was inhibited by 72 h exposure to alpha terpineol in a concentration-dependent manner and the potency in the NF-κB translocation and cell survival assays were in the same range (Figure 3a). The 2 h exposure to celastrol (2.5 μM) and the 4 h exposure to rottelrin (100 μM), MG-132 (20 μM) and 15-delta prostaglandin J2 (10 μM) resulted in 10%, 22%, 27% and 55% of the TNF-α-induced NF-κB translocation, respectively (data not shown). The transcriptional reporter gene assay for the NF-κB pathway revealed that the intracellular status of NF-κB, when activated with TNF-α, in the cervical human cell line ME-180 was inhibited in a dose-dependent manner in response to alpha terpineol treatment (Figure 3b).

Treatment of MCF-7 cells with alpha terpineol for 6 h down-regulated several NF-κB-regulated and related genes such as *IL-1β, IL1R1, IFNG, ITK, EGFR, AKT1S1, TNIK, TRFDD1, BAG1* and *BAG3,* with fold change ranging between 0.05-0.6.

The kinase profiling performed, using a cell free system, in the 50 kinase panel showed that at low concentrations (0.64 mM) the inhibitory activity of alpha terpineol was mostly against MAPK8 (JNK1) (43%). However, alpha terpineol at 5.8 mM was found to moderately inhibit JAK3 (48%), AKT1 (35%) and IKBKB (23%) in a dose-dependent manner (data not shown).

Discussion

In the current study, the different approaches used suggest that alpha terpineol inhibits growth and induces cell death in tumour cells through a mechanism that involves inhibition of NF-κB activity. Among the 14 human tumour cell lines representing different haematological and non-haematological malignancies, alpha terpineol showed significant cytotoxic activity in the small cell lung carcinoma cell line, suggesting tumour-specific activity. The relatively limited effects of the classical mechanisms of resistance represented in the panel on the cytotoxic efficacy of alpha terpineol make the compound potentially interesting for patients with drug-resistant tumours. The slight tumour selectivity of alpha terpineol suggests a decrease risk of toxicity against normal lymphocytes; this is an encouraging

characteristic for this compound as a lack of cytotoxic selectivity between malignant and normal lymphocytes is a feature shared by many of the clinically used cytotoxic drugs (21). Treatment with alpha terpineol induced cell cycle arrest and apoptosis in the cell line tested in a dose- and time-dependent manner. The results further suggest that cell cycle phase arrest by alpha terpineol may depend on drug concentration at the shorter exposure time. This finding is consistent with the only published report on the cell cycle and apoptotic effects of alpha terpineol (3), which showed that this compound is active in inducing cell cycle changes if combined with linalyl acetate rather than if used alone in colorectal tumour cells.

Drug activity patterns in a panel of 10 cell lines had previously shown the possibility to classify anticancer drugs according to the mechanism of action (12). The gene expression signature-based screening approach, the Connectivity Map, has been shown to predict the molecular actions of novel therapeutic compounds accurately and to suggest ways in which existing drugs can be newly applied to treat diseases such as cancer (16, 22). The Connectivity Map approach is based on the similarity of a compound-induced signature to signatures of existing drugs with known mechanism. Using these two approaches, an attempt was made to predict the mechanism of action of alpha terpineol. The findings that the majority of the identified agents which had previously been described in literature to act through inhibition of NF-κB/ protein kinase, mainly AKT kinase and calmodulin, suggest similar mechanism(s) of action to alpha terpineol. The activity profile of alpha terpineol in the cell line panel was very similar to that of linalyl acetate as evident by the very high correlation coefficient between the two substances. There have been previous reports on the anticancer activity of linalyl acetate, as a component of *Salvia libanotica* extract, and its potential as an NF-κB/ protein kinase inhibitor (4). Inhibitors of calmodulin have been reported to prevent the NF-κB activation (23, 24). The connectivity list also included H1-receptor antagonists and a cardiac glycoside. Interestingly, constitutive H1-receptor-mediated NF-κB activation has been shown to be inhibited by all the clinically used H1-antihistamines tested to date (25) and NF-κB inhibition has been suggested as a cytotoxic effect of several structurally related cardiac glycosides in cancer cells (26, 27). Although there is no report on the mechanism of action of lanatoside C in cancer cells, it is interesting to note that the activity pattern of lanatoside C in the 10 cell line panel was previously shown to correlate with the activity pattern of other structurally related cardiac glycosides as ouabain, digoxin and digitoxin (28). The data of the present study thus suggested that alpha terpineol has NF-κB- and protein kinase-inhibiting properties, which warranted further evaluation. In an attempt to validate this hypothesis, subsequent preliminary mechanistic studies were performed.

Activation of the transcription factor NF-κB has been demonstrated to be a link between chronic inflammation and development of cancer and is widely known to induce expression of diverse target genes involved in immune responses, inflammation, cell survival, and cancer (29, 30). An important finding in this study is that alpha terpineol can inhibit the NF-κB translocation and activity in a dose-dependent manner in tumour cells as demonstrated by the two NF-κB assays performed to validate the hypothesis of the present study. Another important finding in this study is that the microarray analysis identified that the expression of several NF-κB-regulated and related genes such as *IL-1β, IL1R1, IFNG, ITK, EGFR, AKT1S1, TNIK, TRFDD1, BAG1* and *BAG3* was down-regulated in response to alpha terpineol treatment. The gene expression of these genes was reduced by less than half-fold. Interestingly, alpha terpineol has previously been described to have anti-inflammatory activity (7), which further suggests possible involvement of NF-κB in its action.

When investigating a possible effect of alpha terpineol on kinases, modest inhibitory activity was observed on JNK1, JAK3, AKT and IKKbeta kinases in the cell-free assay utilised. No or marginal effects were observed on the other kinases in the panel tested when very low concentrations of alpha terpineol were used. This observation may be due to the following two reasons: (i) the cell-free assay environment prevented the interaction between alpha terpineol and certain molecules required for kinase inhibition, or (ii) kinase inhibition may not be one of the major mechanism(s) by which alpha terpineol exerts its cytotoxicity. Still, the moderate inhibition of AKT and IKKbeta kinases may contribute to the observation of the correlation of alpha terpineol with AKT kinase and NF-κB inhibitors.

Disruption of the mitochondrial membrane potential and subsequent release of cytochrome *c* cannot be excluded as an additional mechanism for alpha terpineol cytotoxicity. It has been shown recently that disruption of mitochondrial membrane potential and subsequent release of cytochrome *c* are the reasons for apoptosis induction by the combination of alpha terpineol with linalyl acetate and camphor, the two other active Lebanese sage components, in colon cancer cell lines (3). Disruption of the cell membrane and the abnormal structure of mitochondria of the fungi have been used to explain the antifungal action of alpha terpineol (31). Interestingly, linalyl acetate, thapsigargin, benzethonium chloride, methylbenzethonium chloride and chlorhexidine, which were strongly correlated to alpha terpineol activity in the current study, have been shown to be associated with the loss of mitochondrial membrane potential and release of cytochrome *c* (18-20).

Taken together, these results suggest that alpha terpineol inhibits growth and induces cell death in tumour cells

through a mechanism that involves inhibition of NF-κB activity. However, the observation that many other compounds with different mechanisms of action have correlated well with the activity of alpha terpineol suggests that this compound may have more than one mechanism of action responsible for its cytotoxic effects. The main target of alpha terpineol in the NF-κB signalling pathway is currently the focus of a follow-up study (Sally J Deeb, Saadia B Hassan, Rolf L Larsson and Hala U Gali-Muhtasib: The sage components linalyl acetate and alpha terpineol enhance cell death through inhibition of nuclear factor kappa-B signalling; submitted for publication to Anticancer Drug, 2010)

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