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Gene expression signature-based chemical genomics and activity pattern in a panel of tumour cell lines propose linalyl acetate as a protein kinase/NF-ϰB inhibitor

Research Article

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Abbreviations: American Type Culture Collection, (ATCC); chronic lymphocytic leukaemia, (CLL); fluorescein diacetate, (FDA); Fluorometric Microculture Cytotoxicity Assay, (FMCA); glutathione, (GSH); heat-inactivated fetal calf serum, (FCS); inhibitory concentration 50%, (IC_{50}); multi-drug resistance, (MDR); multidrug-resistance protein, (MRP); normal mononuclear cells, (PBMCs); Nuclear factor-KB, (NF-ϰB); P-glycoprotein 170, (Pgp); phosphate-buffered saline, (PBS); robust multi-array average, (RMA); survival index, (SI); topoisomerase II, (TopoII); Tumour Necrosis Factor-α, (TNFα)

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Summary

The essential oil of Lebanese sage, Salvia libanotica, was reported to have anti-tumour activity; however, the mechanism of action has not been identified yet. In this study, 14- cancer cell lines including drug-sensitive and resistant lung, leukaemia, and colon, as well as primary human tumours of chronic lymphocytic leukaemia (CLL) and primary normal mononuclear cells (PBMCs) were used to characterize the anti-tumour activity and mechanism of action of linalyl acetate, a component of the Lebanese sage essential oil. Drug activity and gene expression data sets were utilized to identify drugs with similar activity patterns and genes involved in drug sensitivity/resistance. In addition, the Connectivity Map, a gene expression signature-based screening approach, assisted in predicting further the molecular action of linalyl acetate. Small cell lung carcinoma and colorectal cancer cell lines were the most sensitive to the drug and greater tumour selectivity was observed against chronic lymphocytic leukaemia cells compared to normal mononuclear cells. Only limited effect of some of the classical mechanisms of multi-drug resistance on the activity of Linalyl acetate was noted which makes it potentially interesting for drug-resistant patients. There was high similarity between the activity-pattern/gene expression profile of linalyl acetate and that of protein kinase/NF-ϰB inhibitors. Validating this, linalyl acetate was found to strongly inhibit Janus kinase, JAK3, and p38α kinases in a cell-free assay as well as the NF-ϰB translocation in a dose-dependent manner. Taken together, our results show that the NF-kB inhibitor, linalyl acetate, may represent a new therapeutic compound in the management of inflammation and cancer.

I. Introduction

Herbs have been considered natural and valuable sources for anti-cancer drug discovery. Nowadays, many of the drugs that have been used for treatment of malignant diseases are derived from natural products. The genus Salvia, a herbal plant comprised of numerous species, has shown diverse biological activities including anti-tumour activities (Liu et al, 2000; Fiore et al, 2006). Water extracts of Salvia libanotica (also known as Salvia triloba or three-lobed sage), a plant endemic to the Eastern Mediterranean, have been used by local populations to cure many health problems such as abdominal pain, indigestion, headaches and respiratory problems (Gali-Muhtasib et al, 2000). The oil extract of this plant has

strong anti-microbial properties (Hilan, 1997) and was found to suppress the formation of skin papillomas in a mouse model as well as exert anti-inflammatory effects on the skin of treated mice (Gali-Muhtasib and Affara, 2000).

Twelve components were identified in the essential oil of Salvia libanotica (Farhat et al, 2001), three of which were found to exert potent anti-proliferative effects on a panel of cancer and normal cell lines (Hala Gali-Muhtasib unpublished data). Linalyl acetate, one of those three components, belongs to the terpenoid family of compounds. This compound is the acetate ester of the unsaturated acyclic monoterpene alcohol, the linalool (**Figure 1**). Linalyl acetate is the principal component of many essential oils from herbal plants such as lavender, cardamom and common thyme. Linalyl acetate has been shown to be cytotoxic to human skin cells, endothelial cells and fibroblasts (Prashar et al, 2004) and has shown, in combination with two sage components, to have anti-tumour activity against colon cancer cell lines (Itani et al, 2008). In addition, linalyl acetate has been ascribed anti-inflammatory (Peana et al, 2002), anti-bacterial (Trombetta et al, 2005), and anti-fungal activities (Barra et al, 2007) and it is used in aromatherapy and in the flavouring and fragrance industries.

In an effort to characterize the anti-tumour activity of new substances and to elucidate their mechanism of action several approaches have been proposed. Drug-response analysis in a panel of cell lines has proven to be an important tool in anti-cancer drug discovery and early evaluation. The patterns of drug activity across cell lines can provide information on mechanisms of drug action (Paull et al, 1989; Boyd, 1995; Dhar et al, 1996). The development of molecular technologies, such as expression micro-arrays, has made it easier to identify genes involved in chemo-sensitivity, while integration of gene expression and drug activity data sets can identify genes involved in drug sensitivity or resistance to specific drugs (Scherf et al, 2000; Rickardson et al, 2005). Recently, the gene expression signature-based screening approach, the Connectivity Map, has been developed as a new tool that relies on genes to connect diseases with potential therapeutic drugs and to predict how new drugs function in cells. The map has the ability to accurately predict the molecular actions of novel therapeutic compounds and has also shown that genomic signatures can be used to recognize drugs with common mechanisms of action as well as to discover unknown mechanisms of action of existing drugs (Hieronymus et al, 2006; Lamb et al, 2006; Wei et al, 2006).

In this study, we characterized the anti-tumour activity of linalyl acetate and investigated its mechanisms of action.

II. Material and Methods

A. Cell line cultures

The panel of ten human cancer cell lines has been described previously (Dhar et al, 1996). 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-resistant sub-lines 8226/Dox40, 8226/LR5, CEMVM-1, U937-vcr, H69AR and the primary resistant ACHN (renal adenocarcinoma). All cells were grown in culture medium RPMI-1640. Four colorectal cell lines, HCT-8, SW620, HCT-116 and HT-29 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in RPMI-1640, Leibovitz's L-15, McCoy's and Dulbecco's Modified Eagle's media, 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.

B. Primary human tumour and normal mononuclear cells

Tumour cell samples were obtained from two patients with chronic lymphocytic leukaemia (CLL). The patient samples were obtained from peripheral blood sampling and leukaemia cells were isolated from the blood by density gradient centrifugation on 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. Cell culture medium RPMI 1640 (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.

C. Linalyl acetate concentration and drug-plate preparation

Linalyl acetate (Acros, Belgium) was tested in duplicate at 8 concentrations from a maximum of 50 mM using 2-fold serial dilutions in phosphate-buffered saline (PBS; pH 7.4 Hyclone).

384-well microtitre plates (Nunc, Roskilde, Denmark) were prepared with 5 μl drug solutions at 10 times the final drug concentration using the pipetting robot BioMek 2000 (Beckman Coulter, Fullerton, CA).

D. Viability assays

Tumour cells from the cell line panel (5000 cells per well), and from CLL and PBMCs (50000 cells per well), were seeded separately in the drug-prepared 384-well plates using the pipetting robot Precision 2000 (Bio-Tek Instruments Inc., Winooski, VT, USA). Three columns without drugs served as controls and one column with medium only served as blank.

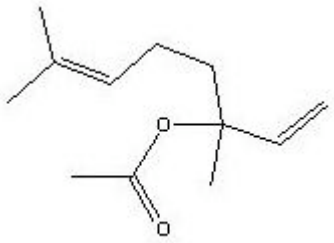

Figure 1. Linalyl acetate structural formula.

The plates were incubated at 37°C for 72 h and were then analyzed using the fluorometric microculture cytotoxicity assay (FMCA). Three repeated experiments were performed where cell preparation, drug preparation as well as FMCA analysis were performed independently.

E. The fluorometric microculture cytotoxicity assay (FMCA) and quantification of results

The fluorometric microculture cytotoxicity assay (FMCA), described in detail previously (Larsson et al, 1992; Rickardson et al, 2005), was performed to measure cell survival. The method is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes. After 72 h incubation at 37°C, medium and drugs were aspirated, the cells were washed twice with PBS, 50 µl of physiological buffer and 1 µl of 0.5 mg/ml FDA were added and after 50-70 min of incubation, the fluorescence, which is proportional to the number of living cells, was measured at 485/520 nm in the FLUOstar Optima. Cell survival was presented as survival index (SI) defined as fluorescence in test wells as a percentage of that in 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 signal in the blank wells.

F. FMCA data evaluation

Data from all three experiments were processed using GraphPad Prism (GraphPad Software, Inc. San Diego, CA) with non-linear regression to a standard sigmoidal dose-response model. Maximum effect and baseline were set at 0% and 100% cell survival, respectively, and IC_{50} (inhibitory concentration 50%) was estimated. When the exposure to the test substance resulted in less than 50% reduction in cell survival in all of the concentration ranges tested, the IC_{50} was set to the lowest concentration used.

G. Drug and gene expression database

A total of 400 standard drugs with known mechanism of action representing different mechanistic groups and investigational substances were tested in the panel of 10 tumour cell lines using the 72 h FMCA, and their IC_{50}s were estimated and included in the database.

The cell lines from the 10 cell lines panel were analyzed for gene expression using cDNA microarrays as described in detail previously (Rickardson et al, 2005). The arrays, which contained 7409 cDNA clones included in the Human Sequence Verified Set, were obtained from Research Genetics (Huntsville, AL, USA). A complete list of genes printed on the arrays is available at http://www.medsci.uu.se/klinfarm/arrayplatform/cDNAgenelist.htm. The drug and gene expression databases were integrated (Rickardson et al, 2005) and a correlation analysis was performed. Pearson's correlation coefficients for all the drug-drug (log_{10} IC_{50}) and drug-gene correlations (log_{10}, log_2) were calculated. A correlation coefficient ≥0.7 was used to identify the drugs that correlated with linalyl acetate activity and ≥ ±0.7 was chosen to extract the genes associated with its resistance and sensitivity. Resistance factors were defined as IC_{50} values in the resistant subline divided by IC_{50} values in the parental cell line.

H. Microarray expression analysis

MCF7 cells were treated with PBS, 25 mM linalyl acetate 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 (NanoDrop Technologies, Wilmington, DE) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA). 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). Affymetrix GeneChip® expression arrays (Human Genome U133 Plus 2.0 Array) were hybridized for 16 h in a 45°C incubator, rotated at 60 rpm. According to the GeneChip® Expression Analysis Technical Manual (Rev. 5, Affymetrix Inc., Santa Clara, CA), the arrays were then washed and stained using the Fluidics Station 450 and scanned using the GeneChip® Scanner 3000 7G.

I. Connectivity Map data analysis

The raw data was normalized using the robust multi-array average (RMA) method (Irizarry et al, 2003). 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 analysis. To investigate the target activity of our compound, we used an approach to connect the activity of linalyl acetate to drugs with known biological activities at the gene expression level (Lamb et al, 2006). The current version of the Connectivity Map data set contains genome-wide expression data for 6100 treatment and vehicle control pairs, representing 1309 distinct small molecule bioactive compounds (perturbagens), www.broad.mit.edu/cmap. Enrichment of both the up- and down-regulated genes from a given signature in the profiles of each treatment instance were estimated as described (Lamb et al, 2006) 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.

K. Kinase profiling

Linalyl acetate kinase profiling was performed, in a cell-free system, against the SelectScreen™ kinase panel of 50 key kinase targets from Invitrogen (SelectScreen Kinome Sampler Panel) at a single concentration of 6.25 mM. The kinases were selected to broadly cover the kinome and to provide a good approximation of compound potency. The assays were performed according to the Screening Protocol and Assay Conditions of Invitrogen www.invitrogen.com. Briefly, the Z'-LYTE™ biochemical assay employs a fluorescence-based, coupled-enzyme format and is based on the differential sensitivity of phosphorylated and non-phosphorylated peptides to proteolytic cleavage.

10-points titration curves in a cell free system, obtained from a maximum of 18.75 mM by 3-fold serial dilution, to identify the IC_{50} of the linalyl acetate inhibitory effect on JAK3, AKT1 and IKKβ activity, were performed. ATP was used at a concentration of 10 µM in all kinase assays except for Kit, MAP2K1, MAPK14, MAPK8 and NTRK1 where the concentration was 100 µM.

L. Nuclear factor-κB translocation

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 linalyl acetate or 2.5 µM celastrol, which was obtained from commercial sources. The plate was incubated for 2 h at 37°C. In the second experiment, linalyl acetate was tested at 5 concentrations from a maximum of 6.25 mM using 2-fold serial dilutions. Rottlerin, MG-132 and 15-δ prostaglandin J2 were obtained from commercial sources 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.) utilizing the ArrayScan® HCS reader (Cellomics, Inc. USA) as described earlier (Ding et al, 1998). The ArrayScan® reader automatically quantifies the mean nucleus-cytoplasm intensity difference of the amount of p65 immunofluorescence staining in a predefined number of cells (500 cells/well). The mean nucleus-cytoplasm intensity difference was presented as NF-κB translocation %, defined as the mean nucleus-cytoplasm intensity difference in the experimental wells as a percentage of that in control wells.

To determine the cytotoxicity of linalyl acetate in parallel to NF-κB translocation analysis, 96 -well microtiter plates (Nunc, Roskilde, Denmark) were prepared with 20 μl per well of drug solution at ten times the desired concentration. Linalyl acetate 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 and at the end of the incubation period, the cell survival was determined with the FMCA as described above.

III. Results

Among the 14 cell lines tested, the small cell lung carcinoma cell line, NCI-H69, and the colorectal cancer cell lines, HCT-116 and HCT-8, were the most sensitive to linalyl acetate (IC_{50} was set to 0.2 mM, see Materials and Methods). The small cell lung carcinoma subline, H69AR, and the myeloma RPMI 8226/S cell lines were also sensitive ($IC_{50:}$ 0.8 and 2.4 mM, respectively) (**Figure 2a**). The IC_{50} in mM of the remaining cell lines were as follows: CCRF-CEM (4.3), CEMVM-1 (4.5), U937-GTB (4.8), SW620 (4.9), U937-vcr, (5.7), ACHN (7.1), 8226/Dox40 (7.9), 8226/LR5 (8.8) and HT-29 (16.4). Linalyl acetate showed higher activity in tumour cells from chronic lymphocytic leukaemia patient samples (CLL) than in normal mononuclear cells from healthy donors (PBMCs) (**Figure 2b**). The tumour selectivity ratio, the ratio between the mean IC_{50} in PBMC and CLL for linalyl acetate, was found to be around 6.

The cytotoxicity of some of the standard agents confirmed the known resistance pattern of the drug resistant sublines (Lindhagen et al, 2007) (**Table 1**). Overall, the resistance factors for linalyl acetate were low. The greatest difference in sensitivity between a parental line and its subline was observed for H69 and its subline H69AR with a ratio exceeding 4. Resistance factors around 3 were observed for resistance associated with Pgp and GSH.

With respect to log IC_{50} patterns, the closest matches to linalyl acetate activity found in the drug activity database were the cardiac glycoside, lanatoside C, the tyrosine kinase inhibitor, PKC412 and the phenothiazine derivative, calmodulin inhibitor, thioridazine. The cardiac glycosides and the calmodulin inhibitors are reported to inhibit NF-κB while PKC412 is a known multi-target kinase inhibitor. In addition, linalyl acetate showed correlations to compounds whose mechanism of action is not clearly defined (**Table 2**). The clinically used agents vincristine, melphalan, cytarabine, topotecan and doxorubicin generally showed lower negative correlations compared to the activity pattern of linalyl acetate.

Using the drug activity-gene expression correlations, 83 genes were identified for linalyl acetate that correlated to resistance (r>0.7) and 160 correlated to sensitivity (r<-0.7). For the genes identified, the distribution of Gene Ontology (GO) terms was investigated using a hypergeometric test (one-sided p value<0.05) (Ameur et al, 2006). In terms of associated biological processes, both groups of genes correlating to resistance and sensitivity showed an over-representation of several processes. Considering the genes associated with these over-represented biological processes there were 68 genes for linalyl acetate sensitivity (supplement 1). These biological processes involved cell cycle, mitotic cell cycle, DNA repair and negative regulation of cell proliferation.

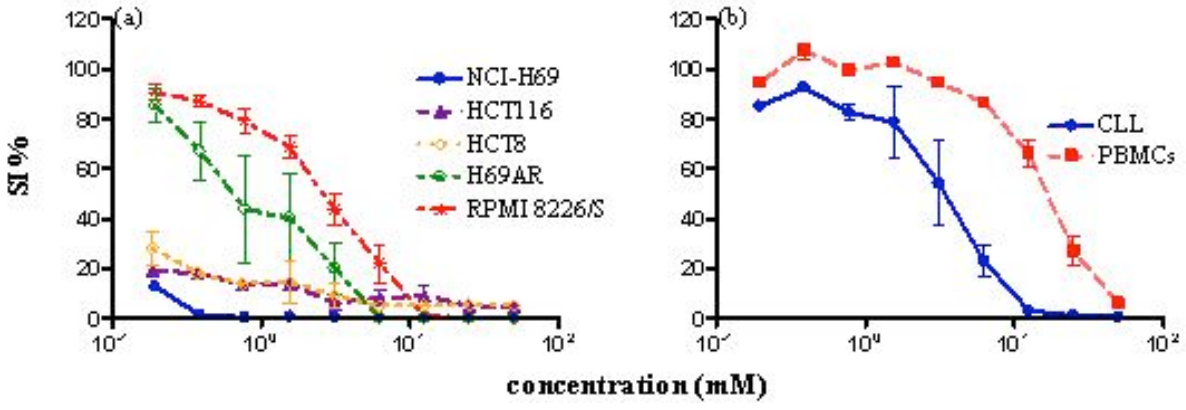

Figure 2. Effect of linalyl acetate, as individual dose response curves assayed by the FMCA, on: (**a**) the small-cell lung, the colon and the myeloma human cancer cell lines (**b**) chronic lymphocytic leukaemia, CLL, (n=2) and normal mononuclear cells, PBMCs, (n=2). Data are represented as mean survival index ± SEM.

Table 1 Influence of resistance mechanisms on cytotoxic potency of linalyl acetate, and five standard cytotoxic agents in the cell line panel.

	TopoII-associated MDR	**MRP-associated MDR**	**Pgp-associated MDR**	**GSH-associated MDR**	**Tubulin-associated MDR**
Parental cell line	CCRF-CEM	NCI-H69	RPMI 8226	RPMI 8226	U937-GTB
Resistant subline	CEM/VM-1	H69AR	8226/Dox40	8226/LR-5	U937/Vcr
Resistance factor					
linalyl acetate	1.0	>4	3.3	3.6	1.2
doxorubicin*	5.6	33	43	0.61	2.0
vincristine*	1.0	27	398	1.1	61
melphalan*	1.1	0.55	3.2	3.5	1.6
cytarabine*	1.0	1.0	1.0	0.03	0.89
cisplatin*	0.71	0.89	4.7	1.1	1.4

The resistance factor was defined as the IC_{50} value in the resistant subline divided by that in its parental cell line. TopoII; topoisomerase II, MDR; multi-drug resistance, MRP; multidrug-resistance protein, Pgp; P-glycoprotein 170, GSH; glutathione.
* Resistance factor values were from (Lindhagen et al, 2007).

Table 2 Correlation between the activity pattern of linalyl acetate and drugs from the drug database with Pearson's correlation coefficient > 0.7 and 5 of the standard cytotoxic drugs and their proposed mechanism of action.

drug	**Classification**	**Pearson's correlation coefficient**
lanatoside C	cardiac glycoside, NF- κB inhibitor, anti-inflammatory	0.91
PKC412	protein kinase inhibitor	0.85
acetochlor	herbicide	0.76
cetrimonium	antifungal	0.74
thioridazine	antipsychotic, calmodulin inhibitor, anti-inflammatory, cyclooxygenase 2/lipoxygenase inhibitor.	0.71
vincristine	platinum compound	-0.5
melphalan	alkylating agent	-0.17
cytarabine	antimetabolite	-0.29
topotecan	topoisomerase I inhibitor	-0.44
doxorubicin	topoisomerase II inhibitor	-0.16

Pearson's correlation coefficient between the log IC_{50}s of the ten cell lines is shown.

To further classify the mechanism of action of linalyl acetate, its gene expression profile (signature) was defined in order to use the Connectivity Map database. The Connectivity Map database was used to identify drugs with high similar gene expression profiles to that of linalyl acetate (linalyl acetate query signature is provided as supplement 2). The up- and down-regulated genes obtained from affymetrix expression analysis of linalyl acetate effect on the breast cancer cell line, MCF7, showed association to several biological processes involving apoptosis and cell cycle. To use the Connectivity Map, we loaded the linalyl acetate query signature to the database and executed the query of substances that induce the biological state represented by the linalyl acetate signature. To develop hypotheses on the mechanism of action of linalyl acetate we scanned the first 100 instances, out of the 6100 instances of the Connectivity Map resources, at the top of the list. High connectivity scores were found for multiple instances of a several perturbagens in which protein kinase/ NF-κB and calmodulin inhibition contributes to their anti-tumour activity. Next we examined the rank of the identified perturbages according to the permutation P value (an estimate of the likelihood that the enrichment of a set of instances in the list of all instances in a given result would be observed by chance). Most of the identified perturbagens with high connectivity scores were ranked below 50 out of all 1309 bioactive compounds of the

Connectivity Map resources (permutation P value ranged between 0.00000-0.001). Given these results we considered the 50 highest ranking perturbagens to be likely relevance in the hypothesis generation process. Analysis of the 50 highest ranking perturbagens resulted in identification of many other agents that protein kinase/ NF-κB inhibition contributes to their anti-tumour activity. Generally, JAK, p38 MAPK, AKT1 and IKK kinases were shown to be inhibited by these perturbagens (**Table 3**).

To test the hypothesis that linalyl acetate is acting on the protein kinase and/or NF-κB pathways, in vitro protein kinase profiling and an NF-κB assay were performed.

The single concentration percent inhibition determinations of each kinase are presented in **Table 4**. Linalyl acetate was found to strongly inhibit JAK3, MAPK14 (p38α), NEK1 and showed a tendency to inhibit other kinases including AKT1, IKKβ and MAPKAPK2 kinases. The concentration- inhibition effect curves of the linalyl acetate on JAK3, AKT1 and IKKβ activity in a cell-free system are displayed in **Figure 3a**. The percent inhibition in the activities of these kinases with linalyl acetate concentrations had the tendency to have the classical sigmoid shape which was more evident with JAK3 activity although the titrations were not carried out to saturation under the concentration range tested. The IC_{50} value of the inhibitory effect of linalyl acetate on JAK3 kinase was 0.8 mM.

The NF-κB assay showed that exposure of Hela cells to linalyl acetate inhibited TNFα-induced NF-κB translocation into the nucleus (**Figure 3b**). The 2 h exposure to celastrol (2.5 μM) and the 4 h exposure to rottelrin (100 μM), MG 132 (20 μM) and 15-δ prostaglandin J2 (10 μM) resulted in 10%, 22%, 27% and 55% of the TNFα-induced NF-κB translocation, respectively. The survival of Hela cells was inhibited by 72 h exposure to linalyl acetate. A concentration-dependent decrease in NF-κB translocation and SI % followed by a tendecy to blateau was observed. The decrease in NF-κB translocation seems to be time dependent as well. The potency of linalyl acetate in the NF-κB translocation and cell survival assays were in the same range (**Figure 3b**).

Table 3 The top perturbagens, among the 50 highest ranking perturbagens, identified through the Connectivity Map in which protein kinase/ NF-κB and calmodulin inhibition contributes to their anti-tumour activity.

Connectivity Map name (n)[1]	maximum connectivity score[2]	Rank[3]	description
rottlerin (3)	1.000	17	NF-κB, p38 MAPK, IKK, MAPKAPK2, and Ca/calmodulin-dependent kinase II inhibitor
phenoxybenzamine (4)	0.985	1	calmodulin inhibitor
thioridazine (20)	0.979	6	calmodulin inhibitor, anti-inflammatory
pyrvinium (6)	0.916	2	Akt kinase inhibitor
calmidazolium (2)	0.858	30	calmodulin inhibitor
clioquinol (5)	0.851	23	NF-κB, proteasome inhibitor
MG-262 (3)	0.821	43	NF-κB, proteasome inhibitor
prochlorperazine (16)	0.807	7	calmodulin inhibitor, anti-inflammatory
Gossypol (6)	0.799	33	Akt kinase inhibitor
15-δ prostaglandin J2 (15)	0.789	28	JAK, IκB kinase, NF-κB inhibitor
resveratrol (9)	0.774	46	JAK, IκB kinase, NF-κB inhibitor
trifluperazine (16)	0.759	9	calmodulin inhibitor, anti-inflammatory
fluphenazine (18)	0.748	24	calmodulin inhibitor, anti-inflammatory
tanespimycin (62)	0.728	10	Hsp90 inhibitor
geldanamycin (15)	0.706	8	Hsp90 inhibitor, NF-κB inhibitor
withaferin A (4)	0.700	21	NF-κB inhibitor
valinomycin (4)	0.696	19	Akt, p38 MAPK kinase inhibitor
trichostatin A (182)	0.573	11	HDAC, Akt, NF-κB inhibitor
alvespimycin (12)	0.554	36	Hsp90 inhibitor
vorinostat (12)	0.543	25	HDAC, NF-κB inhibitor
lanatoside C (4)	0.534	15	cardiac glycoside, NF- κB inhibitor
LY-294002 (61)	0.529	12	Akt, PI3, NF-κB inhibitor
helveticoside (4)	0.520	16	cardiac glycoside, NF- κB inhibitor
sirolimus (44)	0.498	39	Akt, NF-κB, mTOR inhibitor
digoxin (4)	0.490	40	cardiac glycoside, NF- κB inhibitor

[1] the number of all treatment instances of the perturbagen (n)
[2] Maximum connectivity score among all treatment instances
[3] Rank according to permutation P value

Table 4 Inhibition of protein kinases activity by 6.25 mM linalyl acetate. Results are presented as % inhibition of kinase (average of duplicate determination). ATP was 10 μM in all assays except for Kit, MAP2K1, MAPK14, MAPK8 and NTRK1 where it was 100 μM.

kinase tested	% inhibition	kinase tested	% inhibition
ABL1	3	MAP4K4 (HGK)	20
ACVR1B (ALK4)	6	MAPK1 (ERK2)	2
AKT1 (PKB α)	23	**MAPK14 (p38 α)**	**61**
AMPK A1/B1/G1	26	MAPK8 (JNK1)	18
AURKA (Aurora A)	20	MAPKAPK2	32
BTK	34	MARK2	1
CDK1/cyclin B	9	**NEK1**	**65**
CHEK1 (CHK1)	15	NTRK1 (TRKA)	-4
CSNK1G2 (CK1 γ 2)	4	PAK4	3
CSNK2A1 (CK2 α 1)	2	PDGFRB (PDGFR β)	-4
DYRK3	4	PHKG2	2
EGFR (ErbB1)	4	PIM1	7
FGFR1	9	PLK1	15
FLT3	7	PRKACA (PKA)	-4
FRAP1 (mTOR)	1	PRKCA (PKC α)	10
GSK3B (GSK3 β)	11	PRKCB1 (PKC β I)	1
IGF1R	4	PRKCG (PKC γ)	13
IKBKB (IKK β)	18	PRKCD (PKC δ)	-1
INSR	0	RET	-3
IRAK4	-2	ROCK1	14
JAK3	**78**	RPS6KA3 (RSK2)	6
KDR (VEGFR2)	2	RPS6KB1 (p70S6K)	13
KIT	-8	SRC	-11
LCK	0	SYK	13
MAP2K1 (MEK1)	15	TEK (Tie2)	15

Negative values indicate that linalyl acetate was inactive.

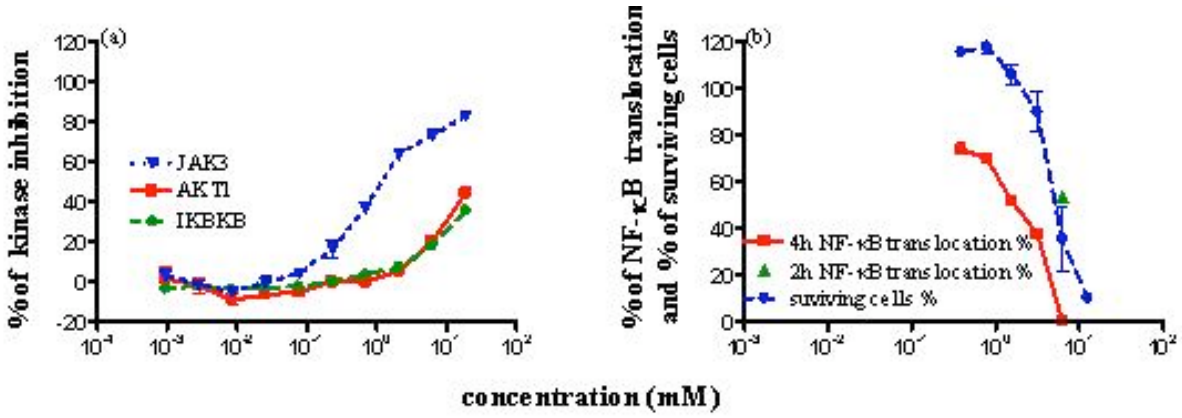

Figure 3. Effect of different concentrations of linalyl acetate on (**a**) JAK3, AKT1 and IKKβ kinases activity in a cell-free system presented as inhibition % (**b**) on the TNFα induced NF-κB translocation and cell survival of the human cervix (Hela) cell line. Data for cell survival are represented as mean ± SEM.

IV. Discussion

The approaches we used in the work presented here identified protein kinases and/or NF-κB inhibition as a possible mechanism for linalyl acetate cytotoxicity. Here we show that linalyl acetate is more cytotoxic, at low concentrations, to the colon and small-cell lung human tumour cell lines than to the other tumour cell lines and is more active against CLL cells compared to PBMC cells, indicating possible tumour selectivity. The potential tumour selectivity of the sage component is interesting, and major toxicity in circulating normal lymphocytes might not be expected if linalyl acetate is tested clinically.

The relatively limited effect of the classical mechanisms of resistance represented in the panel on the cytotoxic efficacy of linalyl acetate makes the compound potentially interesting for patients with drug resistant tumours.

Previous studies on the panel of the 10 cell lines have shown that drug activity patterns can be used to classify anti-cancer drugs according to mechanism of action and this classification matched identifiable patterns of gene expression (Dhar et al, 1996; Rickardson et al, 2005). When comparing the activity pattern of linalyl acetate with the activity of the drugs in the database, the low correlations seen between linalyl acetate and the standard cytotoxic agents from pharmacologically distinct groups suggest that the libanese sage essential oil compound may act by mechanism(s) different from those of clinically established agents. Lanatoside C, PKC412 and thioridazine are agents with response patterns in the cell line panel closely resembling that of linalyl acetate. It has been proposed that NF-κB inhibition is one of the mechanisms through which cardiac glycosides and calmodulin inhibitors exert their cytotoxicity. Structurally related cardiac glycosides such as digitoxin and UNBS1450 have been previously reported to inhibit NF-κB activity in tumour cells (Yang et al, 2005; Mijatovic et al, 2006). There have been no published reports on the mechanism of action of lanatoside C in cancer cells, but interestingly, the activity pattern of lanatoside C in the ten cell lines panel has been shown to correlate with the activity pattern of other structurally related cardiac glycosides such as digoxin and digitoxin (Johansson et al, 2001). Thioridazine, a phenothiazine derivative, is a potent calmodulin inhibitor (Mylari et al, 1990) and phenothiazine derivatives have been reported to have anti-inflammatory activity (Mylari et al, 1990). Inhibitors of calmodulin have been reported to prevent NF-κB activation (Hughes et al, 1998; Shumway et al, 2002). The third agent, PKC412, a staurosporine derivative, is known as a multi-target protein kinase inhibitor that has among its targets AKT kinase (Bahlis et al, 2005). This result thus suggests a potential similarity in mechanism of action between linalyl acetate and these agents.

The gene expression signature-based screening approach, the Connectivity Map developed by Lamb and others, has been shown to accurately predict the molecular actions of novel therapeutic compounds and to suggest ways in which existing drugs can be newly applied to treat diseases such as cancer (Hieronymus et al, 2006; Lamb et al, 2006; Wei et al, 2006). The approach is based on the similarity of a compound-induced signature to signatures of existing drugs with known mechanism. Using this approach, we were able to better predict the mechanism of action of linalyl acetate. The Connectivity Map revealed a strong connection between linalyl acetate and protein kinase/NF-κB inhibitors such as rottelrin, pyrvinium, clioquinol, MG-262, gossypol, 15-δ prostaglandin J2, resveratrol, geldanamycin, trichostatin A, LY-29400 2, valinomycin withaferin A, vorinostat, and sirolimus an observation far beyond that expected by chance (permutation p-value 0.00000-0.001). These inhibitors have been reported to inhibit JAK (Park et al, 2003; Sallman et al, 2007), p38 MAPK (Han and Im, 2008; Ravi et al, 2008) and AKT (Esumi et al, 2004; Chen et al, 2005; Denlinger et al, 2005; Dan et al, 2008; Han and Im, 2008; Moon et al, 2008) kinases as well as the NF-κB (Rossi et al, 2000; Ding et al, 2005; Zavrski et al, 2005; Imre et al, 2006; Yao et al, 2006; Kaileh et al, 2007; Yu et al, 2007; Torricelli et al, 2008; Crevecoeur et al, 2008). In addition, the results demonstrated a strong connection to other substances known as calmodulin inhibitors such as phenoxybenzamine, calmidazolium and the phenothiazine derivatives, thioridazine, prochlorperazine, trifluoperazine and fluphenazine. Three cardiac glycosides, lanatoside C, helveticoside and digoxin were also identified to be best connected to linalyl acetate signature. As discussed earlier in this article, calmodulin inhibitors and cardiac glycosides have been shown to prevent NF-κB activation (Hughes et al, 1998; Shumway et al, 2002; Yang et al, 2005; Mijatovic et al, 2006).

Our data thus suggested that linalyl acetate has protein kinase and NF-κB inhibiting properties, which warranted further evaluation. This hypothesis was confirmed in the subsequent preliminary mechanistic studies performed in this work.

Kinases regulate many different processes such as cell proliferation, differentiation and cell signaling. Our data shows that linalyl acetate had induced strong inhibition of JAK3, NEK and MAPK14 (p38α) kinases in a dose-dependent manner in cell-free system. In addition, linalyl acetate had moderate inhibitory activity to AKT1 (45%) and IKKβ (36%) kinases. Janus kinase (JAK) is an intracellular kinase that regulates signalling pathways activated by a variety of cytokines and its activation results in the activation of the transcription factors STAT (signal transducers and activators of transcription). Several neoplastic diseases including colon cancer have been reported to involve JAK3 activation and targeting JAK3 may be useful for the treatment of colon cancer (Lin et al, 2005; Uckun et al, 2007). MAPK14 (p38α) is another kinase that was inhibited by linalyl acetate. MAPK14 (p38α) is one of the essential kinases for the fate of colorectal cancer cells and its blockade causes growth arrest and cell death (Comes et al, 2007). Interestingly, the colorectal cancer cell lines were the most sensitive tumour cells to linalyl acetate. The concentrations of linalyl acetate that inhibited the protein kinases were in the same range as those that induced cytotoxicity in the different tumour types tested, however in the current study, the protein kinase assays were performed in a cell-free system and it is presently unknown whether the inhibition of the protein kinases is connected to the decreased cell viability. Further experimental data on living cells is needed to have conclusive evidence on protein kinase inhibition by linalyl acetate, and whether linalyl acetate binds specifically to the kinase active-site will be addressed in the future mechanistic studies that are underway in our laboratory.

The NF-κB signalling pathway is a central common regulator for the process of inflammation, and compounds that inhibit different components in the pathway are widely sought as potential therapeutics for inflammatory disorders and cancer (Garg and Aggarwal, 2002). NF-κB is a key antiapoptotic factor in mammalian cells and has a critical role in protecting cells from apoptosis induced by

various anticancer drugs (Baldwin, 2001). NF-κB inhibition may be achieved at various points in the signalling pathway of NF-κB including the downstream kinases (IKKα and IKKβ), the proteosome-mediated degradation of NF-κB inhibitory protein and nuclear NF-κB binding to DNA (Karin et al, 2004). Previously, linalyl acetate has been reported to have anti-inflammatory effects (Peana et al, 2002). Our results indicated an inhibition of NF-κB translocation in response to incubation with linalyl acetate, which seems to be concentration- and time- dependent. Linalyl acetate showed moderate inhibitory activity (36%) against IKKβ kinase in the in vitro, cell-free kinase assay performed in this study. Our study domenstrated that the inhibition of NF-κB translocation was linked to the anti-tumour effect of linalyl acetate in the cell line tested, yet this needs to be explored further and this is the topic of our future paper on linalyl acetate and other sage component modulation of NF-κB pathway.

Disruption of the membrane structure has been used to explain the antimicrobial and the cytotoxic action of linalyl acetate on bacteria and human skin cells (Prashar et al, 2003; Trombetta et al, 2005). A resent study (Itani et al, 2008) has shown that, disruption of mitochondrial membrane potential and subsequent release of cytochrome c are the reasons for apoptosis induction by the combination of linalyl acetate with two other Lebanese sage components in colon cancer cell lines. The same study has shown that treatment with linalyl acetate alone induced PARP cleavage, a feature that known to mediate apoptotic features (Ghobrial et al, 2005). Interestingly, the apoptosis-inducing activity of many of the protein kinase and NF-κB inhibitors that were strongly connected to linalyl acetate activity in the current study, have been shown to be associated with the loss of mitochondrial membrane potential and release of cytochrome c such as rottlerin (Liao et al, 2005), gossypol (Lei et al, 2006), 15-δ prostaglandin J2 (Cho et al, 2006), resveratrol (Aziz et al, 2006), geldanamycin (Nomura et al, 2004), withaferin A (Malik et al, 2007).

The genes from the drug-activity/gene expression correlation approach and the genes from the gene expression signature-based screening approach had diverse functions but a considerable number were found to be related to apoptosis, cell cycle, DNA repair and to the negative regulation of cell proliferation. Interestingly, some of the genes associated with linalyl acetate sensitivity such as the poly (ADP-ribose) polymerase family members PARP2 (Sharkey et al, 2007), nucleophosmin/B23 NPM1 (Lu et al, 1996), the cyclin-dependent kinase inhibitor 1C (p57, Kip2) CDKN1C (Samuelsson et al, 2002) and the genes upregulated by linalyl acetate such as metallothionein MT1 (Chen et al, 2001) and heme oxygenase (decycling) 1 HMOX1 (Das et al, 2006; Lim et al, 2007) have been shown to be linked to the activity of some of the protein kinases/NF-κB inhibitors discussed in this study, or are upregulated by them (supplement 1 and 2).

In conclusion, the approaches used here - correlation analysis in a drug and gene-expression database, gene expression signature-based screening (the Connectivity Map), protein kinase profiling and the NF-κB translocation assay - proposed that protein kinases and/or the NF-κB pathway are targets for linalyl acetate cytotoxicity. This, in addition to all published reports on linaly acetate and sage components cytotoxicity, makes linalyl acetate an interesting compound for cancer therapy especially colon cancer which warrant conducting clinical trials.

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