



Australian Government
**Rural Industries Research and
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Anticancer Activity of Tea Tree Oil

RIRDC Publication No. 10/060



RIRDC Innovation for rural Australia



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Development Corporation**

Anticancer Activity of Tea Tree Oil

by Dr Sara J Greay, Dr Christine Carson, Dr Manfred Beilharz,
Mr Haydn Kissick, Dr Demelza Ireland and Professor Thomas V Riley

with support from Novasel

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Researcher Contact Details

Professor Thomas V Riley
Discipline of Microbiology and Immunology
School of Biomedical, Biomolecular and Chemical Sciences
Faculty Life and Physical Sciences
University of Western Australia

Phone: 08 9346 3690
Fax: 08 9346 2912
Email: triley@cyllene.uwa.edu.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details

Rural Industries Research and Development Corporation
Level 2, 15 National Circuit
BARTON ACT 2600

PO Box 4776
KINGSTON ACT 2604

Phone: 02 6271 4100
Fax: 02 6271 4199
Email: rirdc@rirdc.gov.au
Web: <http://www.rirdc.gov.au>

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Foreword

Cancer is the second leading cause of death worldwide. Malignant mesothelioma and malignant melanoma represent cancers that have a poor prognosis and respond poorly to chemotherapy. Although more than 60% of anticancer agents currently in use are derived from natural resources, toxic side effects and resistance to these and other synthetic drugs necessitates the search for novel agents.

Tea tree oil (TTO), the essential oil from the Australian native *Melaleuca alternifolia* has demonstrated a variety of beneficial efficacies including antimicrobial, antifungal, antiviral and anti-inflammatory. Amongst the activities listed above, anti-cancerous efficacy has also been identified. A single study demonstrated terpinen-4-ol and TTO have *in vitro* anticancer activity. Accordingly, further study of the potential anticancer activity of TTO and its major components is warranted. Currently, no investigation of TTO or terpinen-4-ol has been conducted in *in vivo* studies and before *in vitro* data can be translated clinically, *in vivo* studies must be completed.

The aims of this study were to examine the *in vitro* anticancer efficacy of TTO and its components against cancer and normal cell lines and to examine the *in vivo* efficacy of TTO as a potential antitumor agent using mouse mesothelioma and mouse melanoma models.

This report discusses data obtained on the *in vitro* activity of TTO, specifically by significantly reducing viability of malignant mesothelioma and melanoma cells compared with normal human fibroblasts cells in a dose- and time-dependent manner. This study has demonstrated that TTO and terpinen-4-ol induce their mechanism of action through necrosis, low level apoptosis and cell cycle arrest. TTO induces *in vivo* tumour growth inhibition of both murine subcutaneous malignant mesothelioma and melanoma tumour models studied and induces tumour regression in the mesothelioma tumour mouse model.

This project was funded from industry revenue from Novasel Pty Ltd., which was matched by funds provided by the Australian Government.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Tea Tree Oil R&D Plan 2006-2011, which aims to support studies that demonstrate the efficacy of tea tree oil .

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Tony Byrne
Acting Managing Director
Rural Industries Research and Development Corporation

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Executive Summary

What this report is about

The aims of this study were two-fold. Firstly, to examine the *in vitro* anticancer efficacy of TTO and its components against cancer and normal cell lines by evaluating cytotoxicity by MTT assay, and by further analysing the promising components by examining induction of apoptosis and necrosis and possible cell cycle arrest. Secondly, to examine the *in vivo* efficacy of TTO as a potential antitumor agent using mouse mesothelioma and mouse melanoma models.

Background

Cancer is the second leading cause of death worldwide, accounting for 12% of all deaths and exceeded only by cardiovascular disease. Malignant mesothelioma and malignant melanoma represent cancers that have a poor prognosis and respond poorly to chemotherapy. Although more than 60% of anticancer agents currently in use are derived from natural resources, toxic side effects and resistance to these and other synthetic drugs necessitates the search for novel agents.

Tea tree oil (TTO), the essential oil from the Australian native *Melaleuca alternifolia* has demonstrated a variety of beneficial efficacies including antimicrobial, antifungal, antiviral and anti-inflammatory. Amongst the activities listed above, anti-cancerous efficacy has also been identified. A single study demonstrated terpinen-4-ol and TTO *in vitro* anticancer activity. Accordingly, further study of the potential anticancer activity of TTO and its major components is warranted. Currently, no investigation of TTO or terpinen-4-ol has been conducted in *in vivo* studies and before *in vitro* data can be translated clinically, *in vivo* studies must be completed.

Results

TTO and terpinen-4-ol significantly affect the *in vitro* viability of 2 murine tumour cell lines; AE17 and B16 in a dose- and time- dependent manner as assessed by the MTT assay. This effect is significantly pronounced in AE17 cells with equivalent concentrations of TTO 1.5-1.6-fold more efficacious compared with B16 cells and terpinen-4-ol, 2.5-4-fold more efficacious in AE17 cells compared with B16 cells. In contrast, TTO had a dose- dependent effect against normal human fibroblasts HF32, but this effect was only significantly cytotoxic at doses 2.7-3.5-fold greater (24-72h) compared with AE17 cells and 1.6-1.8-fold greater (24-72h) compared with B16 cells. Again, terpinen-4-ol was significantly less effective against HF32 cells compared with the cancer cell lines and was only cytotoxic to HF32 cells at doses 5-11-fold greater compared with AE17 cells and 2.5-4-fold greater compared with B16 cells.

AE17 cells treated with TTO for 24 and 48h (0.04%) underwent significant necrosis exhibited by cell swelling, nuclear swelling and cell lysis and significant but lower levels of apoptosis. Only with an increased exposure time of 48h and increased concentration to 0.06% was significant necrosis evident in B16 cells, with negligible apoptosis. Consistently, B16 cells demonstrate a higher resistance to terpinen-4-ol than AE17 cells as observed in cytotoxicity measurements using the MTT assay. Due to the low levels of cell death induced, that were inconsistent with cytotoxicity indicated by the MTT assay; other potential mechanisms of action were investigated.

TTO and terpinen-4-ol induced significant cell cycle arrest in both AE17 and B16 cell lines. Specifically, significantly greater cell cycle arrest was observed in B16 cells when compared with AE17 cells with both TTO and terpinen-4-ol. Cell phase arrest appeared to be dose- and time-dependent. With a 12 and 24h exposure of TTO and terpinen-4-ol, a G0/G1 arrest was observed in B16 cells, resulting in the delay of the transition from G0/G1 to S phase which is apparent as a decrease in cells in S phase. This pattern was also apparent in AE17 cells but to a much lower extent. The arrest in G0/G1 was again observed with increased concentrations of TTO but was also coupled with G2/M

phase arrest. At increased exposure times to 48h, TTO induced G2/M arrest. This apparent shift in cell cycle arrest was evident following treatment with TTO in both cell lines cell cycles as TTO reduced cells cycling in G0/G1 compared with control cells in both cell lines, with a slight increase in S phase but concomitant with significant arrest in G2/M phase cells.

The *in vivo* part of the study demonstrated that TTO in a 3% and 10% solution in DMSO and 3% TTO in Novasel® gel applied topically to subcutaneous tumours of malignant mesothelioma and melanoma can induce tumour growth inhibition; and even induce tumour regression (3% and 10% TTO in DMSO) in AE17 tumours. DMSO is known to enhance penetration of substances through the skin. It appears that this formulation is critical to induce tumour regression. Treatment was limited to 4 days of 10% TTO and 16 days of 3% TTO due to significant skin irritation. This skin irritation does however completely resolve following cessation of treatment. Tumours did relapse upon cessation of treatment in the majority of mice in the treatment groups. Whilst reducing TTO concentration from 10% to 3% in DMSO did allow increased treatment time from 4 to 16 days, as soon as treatment was ceased tumours began to regrow. 3% TTO in Novasel® gel does not result in any skin irritation but fails to induce tumour regression. Never has any study reported anti-tumour *in vivo* efficacy of TTO in any model.

Implications

Topical chemotherapy represents a non-invasive, convenient method for treating tumours. Current treatment regimes against skin carcinomas e.g. 5-Fluoruracil and Imiquimod have limited success as tumour recurrence and local and systemic toxicity are problematic. The demonstration that 4 applications of topical TTO can inhibit tumour growth and even induce tumour regression of subcutaneous models of mesothelioma and malignant melanoma with local toxicity in the form of skin irritation completely resolving, is extremely encouraging. If TTO proves to be a clinically useful treatment for cancer, this will be highly significant for the industry and the community. For industry, interest in and demand for the oil would be increased, for the community, a new, inexpensive, widely available anticancer treatment would be most welcome. Corroboration of the anticancer activity of TTO would further enhance the profile of this mainly Australian product and is likely to boost demand for and exports of the oil. Additional effective therapies for cancer would represent an enormous social benefit. Increased demand for TTO would strengthen the industry and the rural communities based on it. Validation of the medicinal applications of TTO will promote use of this natural, renewable resource.

Recommendations

Further studies must work towards a formulation of TTO which is both growth inhibitory and induces tumour regression that is well tolerated. Tumours also resume post cessation of treatment. Future TTO preparations may also need to be prepared such that they can be administered over a longer period of time in order to allow more prolonged tumour growth inhibition and tumour regression. Alternately, a preparation that can induce a complete and long-lived tumour growth regression would be ideal. The *in vivo* mechanisms of action need to be thoroughly investigated, specifically examining possible direct and indirect effects of TTO.

1. Introduction

Cancer is the second leading cause of death worldwide, accounting for 12% of all deaths and exceeded only by cardiovascular disease (Shibuya, 2002). In terms of prevalence, Breast, Prostate, Colorectal and Lung are the most common (Parkin, 2005). In Australia, 83% of all cancers diagnosed are skin cancers, the highest rate worldwide (Australian Institute of Health and Welfare (AIHW) & Australasian Association of Cancer Registries (AACR) 2004).

Malignant mesothelioma and malignant melanoma represent cancers that have a poor prognosis and respond poorly to chemotherapy. Australia has the highest incidence of mesothelioma worldwide (3 times higher than US) (Burkitt, 2007) and the second highest mortality rate of malignant melanoma (Geller, 2007). Current palliative but non-curable chemotherapy for malignant mesothelioma includes cisplatin, gemcitabine and pemetrexed, whilst topical treatment regimes against malignant melanoma e.g. 5-fluorouracil and imiquimod have limited success as tumour recurrence, and local and system toxicity are problematic (Ogbourne, 2004).

Although more than 60% of anticancer agents currently in use are derived from natural resources (Newman, 2003), toxic side effects and resistance to these and other synthetic drugs necessitates the search for novel agents. Current cancer drugs derived from natural products; include Taxol® from the bark of the pacific yew tree, *Taxus brevifolia*, which is one of today's most widely used chemotherapeutic agents. However, toxic side effects and resistance associated with plant and non-plant derived anticancer agents represents a serious problems. Adversities following administration of anticancer drugs include: neurotoxicity, myelosuppression, nephrotoxicity, haemotoxicity, gastrointestinal toxicity, cardiotoxicity, ulceration, nausea, edema, diarrhoea, alopecia, hypersensitivity and anaemia. This highlights the importance of the search for novel chemotherapeutic agents.

Currently, a great deal of research is focusing on the investigation of certain plant species as a source of experimental therapeutic agents, specifically, protodioscin from fenugreek reduced tumours in breast carcinogenic rats (Amin, 2005); curcumin, the major component of turmeric, reduced tumour burden in Ehrlich ascites carcinoma bearing mice (Pal, 2001); whilst green tea extract decreased incidence and size of mouse skin tumours (Wang, 1992).

Of particular interest is ingenol 3-angelate isolated from the sap of *Euphorbia peplus*. It is currently being investigated as a topical chemotherapeutic agent against skin cancer, as a 42nmol dose of an isopropanol based gel for 3 days achieved a 100% cure rate in mice with subcutaneously implanted tumours including B16 melanoma tumours (Ogbourne, 2007). It's mechanisms of action involve primary cell death by necrosis, activation of protein kinase C, the activation of neutrophils *in vivo*, and the induction of cell cycle (G1 and G2/M) arrest.

Cell cycle arrest is a pathway that numerous chemotherapeutic agents interfere with as a mechanism to prevent tumour growth. All cells cycle by a) Gap 1 (G1) phase where high levels of biosynthetic activities take place and G0 phase, where cells are in a resting state (quiescent), until activated to further grow, b) Synthesis (S) phase, where DNA synthesis initiates and upon completion cells enter into c) G2/M; Gap2 (G2) where protein synthesis initiates, upon completion, cells enter into M, Mitotic stage, also known as nuclear division, where the cell that now has 4N DNA (double quantity) splits into two cells each with 2N DNA (normal DNA content). Arrest of cells in any of these cell cycle phases may lead to cell death by apoptosis or necrosis.

Apoptosis or programmed cell death is a highly orchestrated process that induces cellular self destruction and occurs naturally to rid unwanted cells from a given population, without evoking inflammation. The conservative nature of cell death is the outcome irrespective of mode of action of most current anticancer drugs; killing most cancer cells by this cell death type (Kaufmann and Earnshaw, 2000)

However, resistance to cancer agents through drug metabolism, drug target mutation (Gottesman, 2002), compartmentalisation (Kitazono, 1999) and through primary resistance; such as over-

expression of MDR1 (the gene for P-glycoprotein) and especially apoptotic gene mutations of cancer cells, which render them resistant to apoptosis, means the search for novel antitumour agents that may overcome this is warranted.

Necrosis is a form of cell death that occurs as a result of injury which is executed by swelling of the cell, nucleus and mitochondria, followed by membrane rupturing which releases cell contents and invokes inflammation in surrounding tissue. However, if this mode of cell death is induced by chemotherapeutic agents and is coupled with activation of the immune system (an inflammatory response) this may assist in the clearance of tumour and lead to rapid healing and regeneration in the tumour site and surrounding area.

Tea tree oil (TTO), the essential oil from the Australian native *Melaleuca alternifolia* has demonstrated a variety of beneficial efficacies including antimicrobial (Carson, 2006), antifungal (Hammer, 2003), antiviral (Schnitzler, 2001) and anti-inflammatory (Hart, 2000).

TTO consists of over 100 components; of which the major are: terpinen-4-ol, γ -terpinene, α -terpinene and 1,8-cineole. It is the most abundant component, terpinen-4-ol that is the likely mediator of the *in vitro* and *in vivo* efficacy TTO (Mondello, 2006). Amongst the activities listed above, anti-cancerous efficacy has also been identified. A single study demonstrated terpinen-4-ol and TTO *in vitro* anticancer activity. Human melanoma and Adriamycin resistant melanoma cells treated with TTO and terpinen-4-ol underwent caspase dependent apoptosis; a process thought to involve plasma membrane interaction via lipid reorganisation (Calcabrini, 2004). Interestingly, both TTO and terpinen-4-ol were more effective against the resistant cell line suggesting that perhaps neither are substrates for P-glycoprotein, a very useful property in the treatment of MDR (multidrug resistant) tumours. Only one study has demonstrated *in vitro* anticancer efficacy of 1,8-cineole against two human leukaemia cells lines through apoptosis (Moteki, 2002). Accordingly, further study of the potential anticancer activity of TTO and its major components is warranted. Currently, no investigation of TTO or terpinen-4-ol has been conducted *in vivo* and before *in vitro* data can be translated clinically, *in vivo* studies must be completed. In this study we investigate the effect of TTO and components *in vitro* coupled with an investigation of the effects of TTO on tumour development in immuno-competent murine tumour models.

2. Objectives

The objectives of this study are:

1. To examine the *in vitro* anticancer efficacy of TTO and its components against cancer and normal cell lines by evaluating cytotoxicity, and induction of apoptosis and necrosis. Promising components found with *in vitro* testing will be further analysed.
2. *In vivo* testing of TTO and/or its components using mouse mesothelioma and mouse melanoma models. This involves growth of subcutaneous tumours in mice which are treated by topical application and direct injection into the tumour. Systemic delivery of the agents by intra-peritoneal injection may also be investigated.

This study will consist of two parts, examining both *in vitro* and *in vivo* efficacy.

In vitro, an MTT viability assay will evaluate cytotoxicity against cancer and normal cells following treatment with TTO and its components; this also establishes a dose effective concentration range. Fluorescence microscopy using stains such as Hoechst and Annexin V coupled with propidium iodide will allow visualisation of cell death by apoptosis and necrosis; employing flow cytometry to quantify apoptosis/necrosis and; to determine if cell cycle arrest is involved.

An *in vivo* analysis will use murine tumour models to evaluate the efficacy of TTO as an antitumour agent by measuring changes in tumour growth following its application. This *in vivo* analysis using murine tumour models will allow the direct comparison of *in vitro* and *in vivo* effects of TTO for the potential translation of human *in vivo* efficacy in the clinic.

This study will increase our understanding of TTO's anticancer properties, and should a potential anticancer agent/treatment regime be identified, is likely to stimulate further research into TTO and may lead to the development of a novel chemotherapeutic agent for cancer.

3. Methodology

Tissue Culture

Cell maintenance

All cell lines were maintained at 37°C in 5% CO₂ in 75cm³ or 225cm³ tissue culture flasks. AE17 mesothelioma and B16 Melanoma cells were maintained in RPMI-1640 media supplemented with 10% Foetal Calf Serum (FCS), 2mM L-glutamine, antibiotics (50mg/L gentamycin, 60mg/L benzyl penicillin) and 0.05mM 2-mercaptoethanol. HF32 fibroblast cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% FCS, 2mM L-glutamine and antibiotics (5,000 Units/ml Penicillin and 5mg/ml streptomycin).

Sub-culturing cells

AE17, B16 and HF32 cells grow as monolayers. Upon reaching ~60% confluency, all cell lines were sub-cultured using 0.5% trypsin-EDTA (1x) solution. Cell culture medium was aspirated and discarded and cells were washed gently with phosphate buffered saline (PBS) before adding trypsin. Flasks were incubated at 37°C for 1-4 min following agitation by tapping, to harvest all cells. Culture medium was added to the cell/trypsin solution with gentle pipetting to ensure a single cell suspension. Cell suspensions were then centrifuged (500xg) for 5 min, re-suspended in culture medium and an aliquot counted (equal volumes mixed with trypan blue to exclude dead cells) with a haemocytometer under a phase contrast microscope. Cells were then re-seeded into a new tissue culture flask at a 1/10 dilution. For assay, cells were seeded into 96-well plates (1.8x10³/well/100µl), 6-well tissue culture dishes (5x10⁴/1.5ml/well) or 25cm³ tissue culture flasks (3x10⁵/5ml).

Cryopreservation and thawing of cells

Centrifuged cell pellets were gently re-suspended in 10% dimethyl sulphoxide (DMSO) in FCS at 1x10⁶/ml/cryovial. These were subsequently frozen at -80°C in a polystyrene container overnight, then transferred to liquid nitrogen (-196°C).

Frozen cells were retrieved and defrosted in a 37°C water bath. Cells were re-suspended with culture medium and centrifuged at (500xg) for 5 min. Cell pellets was washed once with culture medium, re-suspended in culture medium and added drop-wise to 75cm³ flasks. Following overnight adherence, cells were fed with fresh culture media.

In vivo tumour cell implantation for subcutaneous growth and tumour monitoring

Harvested tumour cells (see sub-culturing cells) were washed twice with PBS, resuspending at concentrations of: 1x10⁷ AE17 cells/100µl/mouse and 5x10⁵ B16 cells/100µl/mouse.

Using a clean sterile 100µl Hamilton syringe (26 ½ gauge needle), 100µl of adequately mixed cell suspension was aspirated and slowly injected sub-cutaneously by holding the mice in the intraperitoneal position.

Tumour development was monitored visually and physically by palpating the tumours/tumour implantation area. Tumours were measured using a microcaliper to obtain 2 tumour diameters taken at right angles to each other; these are multiplied to obtain tumour area (mm²).

Simultaneously, mice condition was monitored daily for any loss of condition by checking for: hunching, ruffled coat, loss of body weight, bloating, slowed movement and seclusion.

Humane culling by Penthrane inhalation followed by cervical dislocation was carried out when tumour area reached 100mm², if tumour has erupted in any way, or if an overall loss of condition was apparent.

Tea Tree Oil

Melaleuca alternifolia (Tea tree) oil (TTO) was kindly provided by P.Guinane Pty. Ltd. Batch 1216 was used for all studies and had the composition as shown (Table 1) evaluated by gas-chromatography mass spectrometry carried out by NSW Department of Primary Industries, Diagnostic and Analytical Services, Environmental Laboratory, Wollongbar, NSW.

Table 1: Composition of *Melaleuca alternifolia* oil batch no. 1216

Components			Percentage		
Components		Percentage	Components		Percentage
1.	α -pinene	2.4	9.	terpinen-4-ol	42.4
2.	sabinene	0.3	10.	α -terpineol	3.1
3.	α -terpinene	9.0	11.	aromadendrene	1.1
4.	limonene	1.1	12.	ledene	0.6
5.	p-cymene	3.1	13.	δ -cadinene	0.8
6.	1,8-cineole	3.7	14.	globlrol	0.2
7.	γ -terpinene	20.1	15.	viridiflorol	0.1
8.	terpinolene	3.2			

Treatments

For *in vitro* assay, TTO and components were made up fresh by mixing equal volumes with DMSO (26 μ l of each). Following this, 44 μ l of the mixture was added to 11ml of warm supplemented tissue culture medium with vigorous vortexing; this yielded a 0.2% solution which was kept in 37°C water bath until ready to dilute further in tissue culture media.

For *in vivo* application, TTO was diluted in DMSO and aliquots were stored frozen -20°C until ready for use.

Staurosporine was made as a stock solution of 1mM in DMSO and stored at 4°C.

“Mock” TTO was made by mixing the following major components of TTO at concentrations equivalent to their composition as listed above (Table 1); 40% terpinen-4-ol, 20% γ -terpinene, 10% α -terpinene 5% 1,8-cineole and 5% p-cymene, 20% Ethanol (EtOH) was included to total the solution composition to 100%.

In vitro analysis

MTT 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide assay

Cellular viability was assessed using the MTT assay (with slight modifications), in which the yellow tetrazolium salt is reduced by the mitochondrion of live but not dead cells to form a purple formazan product, which is solubilised and measured spectrophotometrically (Mosmann, 1983) (Plumb, 1989). Cells subcultured in 96-well plates (6 replicates) with overnight adherence, were treated with concentrations (0.005-0.2%) of TTO, terpinen-4-ol, “Mock TTO”, γ -terpinene, α -terpinene, 1,8-cineole or p-cymene in tissue culture media (150 μ l/well) for 24-72h. Following this, 50 μ l of MTT solution (5mg/ml (PBS) sterile filtered) was added to each well and incubated for 3-6h at 37°C. The solution was then gently aspirated, and 160 μ l of 10% sodium dodecyl sulphate (SDS) (dH₂O) was added to each well following an overnight incubation at 37°C to dissolve the formazan crystals. Absorbance was measured in a microplate reader using a filter at 570nm.

Results are expressed as a percentage of the solvent control wells. IC₅₀ values (Inhibition concentration eliciting 50% inhibition) were determined by linear and polynomial regression.

Visualisation of apoptotic and necrotic cells by fluorescence microscopy

22mm² coverslips were sterilised by soaking in 70% EtOH for 30sec and air-drying for 30min. Following this, the sterile coverslips were added to 6-well tissue culture dishes, cells (5×10^4 /1.5ml) were added and incubated overnight.

Attached cells were treated with TTO, terpinen-4-ol or staurosporine (positive control) and incubated for 48h. Following this, any floating cells were collected by centrifugation, and washed twice with PBS. Cells were then triple stained with Hoechst 33343, Annexin V-Fluorescein isothiocyanate (FITC) and Propidium Iodide (PI) by resuspending cell pellets in 20µl of 1x Annexin V-FITC binding buffer (Biovision) containing 2µl of Annexin-V-FITC (Biovision), 1µl PI (1mg/ml) and 0.5µl of Hoechst 33343 (1mg/ml/PBS) and added to microscope slides. Corresponding coverslips with washed attached cells were then inverted, added to the slides and stained for 15min in the dark. Cells were analysed by fluorescence microscopy (40x) using a triple filter where brightly fluorescing Hoechst 33343 stained cells displaying shrinkage with condensed, segmented nuclei were deemed apoptotic (He, 2000) cells that have bound Annexin V-FITC with green staining in the plasma membrane were deemed apoptotic whilst cells that have lost membrane integrity with red staining (PI) throughout the nucleus and cells that were stained with both Annexin-V-FITC and PI were deemed necrotic.

Quantification of apoptosis and necrosis

Apoptosis and necrosis following treatments was quantified by flow cytometry (FACS) using an Annexin-V-Phycoerythrin (PE) apoptosis detection kit from Becton Dickson Pharmingen™ according to the manufacturer's instructions with slight modifications. Cells cultured in 25cm³ tissue culture flasks and treated with "Mock" TTO, TTO, terpinen-4-ol or staurosporine for 24 and 48h were harvested via trypsinisation, included with any floating cells, centrifuged and resulting pellets washed twice in ice-cold PBS (5% FCS). Cell pellets were then resuspended in 100µl 1x Binding buffer containing 1µl Annexin-V-PE and 1µl 7-Amino-actinomycin (7-AAD) in fluorescence-activated cell sorting (FACS) tubes. Cells were gently vortex and incubated for 15min at 25°C in the dark. Following this, 400µl of 1x binding buffer was added to each tube and were analysed using a BD FACSCanto™ benchtop flow cytometer where Annexin-V-PE positive cells were deemed apoptotic whilst 7-AAD and 7-AAD/Annexin-V-PE positive cells were deemed necrotic. A minimum of 10,000 events were quantified for each treatment and were analysed using FlowJo to calculate % apoptosis and necrosis in the cell populations.

Analysis of cell cycle distribution

Cells cultured in 25cm³ tissue culture flasks and treated with "Mock" TTO, TTO, terpinen-4-ol or staurosporine for 12, 24 and 48h were harvested via trypsinisation, included with any floating cells, centrifuged and resulting pellets washed twice in ice-cold PBS. Cell pellets were then resuspended in 300µl ice cold PBS and fixed in ice-cold 70% EtOH added drop wise with vortexing following overnight incubation at 4°C. Following this, cells were centrifuged (500xg for 4 min) and washed once with 1ml PBS. Cell pellets were resuspended in 500µl of PBS containing 1µl RNase A to give a final concentration of 200µg/ml. Cells were then incubated at 37°C for 40min, transferred to FACS tubes and 2.5µl of PI solution (PBS) was added to give a final concentration of 10µg/ml. Cells were then vortexed and analysed using a BD FACSCanto™ bench top flow cytometer. A minimum of 10,000 events were quantified for each treatment. Percentages of cell cycle distribution in phases: G0 (quiescent/resting phase) /G1(gap phase 1), S (DNA synthesis phase) and G2 (gap phase 2)/M(Mitosis phase) were calculated in only (2N (normal DNA content)/ 4N(double DNA content)) gated cells by DNA content analysis using the Dean-Jett-Fox model in FlowJo software and are compared with control, untreated cells.

***In vivo* antitumour analysis**

C57BL/6J mice bearing established subcutaneous tumours of AE17 Mesothelioma (~9mm²) or B16 Melanoma (~15mm²) or non-established (~0mm²) B16 Melanoma, with shaved tumour/ tumour surrounding area were treated:

- Topically daily for 4 days by pipetting 50µl DMSO alone (solvent control) or 50µl of 10% TTO in DMSO rubbing with a gloved finger to the tumour and surrounding tumour area.
- Topically daily for 13 (B16) or 16 (AE17) days by pipetting 50µl DMSO alone (solvent control) or 50µl of 3% TTO in DMSO or 50µl of 3% TTO Novasel® gel rubbing with a gloved finger to the tumour and surrounding tumour area.
- Topically twice daily for 8 days (B16) by pipetting 50µl of 0% TTO Novasel® gel (control), 50µl of 5% TTO Novasel® gel or 50µl of 5% TTO Novasel® after-wax cream rubbing with a gloved finger to the tumour and surrounding tumour area.
- Topically once daily for 10 days (B16) by adding ~ 50µl of 0% (control), 5%, 10% or 15% TTO Novasel® gel rubbing with a gloved finger to the tumour and surrounding tumour area.

Tumour growth was measured daily as described (*In vivo* tumour cell implantation for subcutaneous growth and tumour monitoring) and results compared tumour growth of control mice with TTO treated mice.

Statistical Analysis

All data are presented as the mean± S.E.M (standard error of the mean) or ±S.D (Standard deviation). Statistical analysis was conducted by Student's t-tests using Microsoft Excel with F-tests to determine unequal or equal variance of the means of the populations. Significant P-values of <0.05 were calculated by two-tailed test when comparing differences between controls and treatments or by one-tailed tests to compare increases in apoptosis or necrosis compared with controls at a 95% confidence interval. All graphs were plotted using Microsoft Excel.

4. Results

In vitro experiments

Tea Tree Oil inhibits cellular viability

TTO inhibited cellular viability of the two tumour cell lines studied AE17 Mesothelioma and B16 Melanoma, in a dose-and time-dependent manner as measured by the MTT assay (Table 2 and Figures 1 and 2).

TTO was most efficacious against the AE17 cells with IC_{50} values of $0.03\% \pm 0.005$ (24h), $0.02\% \pm 0.006$ (48h) and $0.02\% \pm 0.003$ (72h) (Table 2). Increasing exposure time from 24h to 48h significantly ($P < 0.05$) decreased AE17 cellular viability, with concentrations as low as 0.01% TTO (after 48h) significantly reducing viability to 70% compared with control cells (Figure 1).

Concentrations of TTO (0.02-0.1%) after 24h, (0.01-0.1%) after 48h and (0.02-0.1%) after 72h all significantly ($P < 0.05$) reduced AE17 cell viability (Figure 1).

TTO significantly reduced viability of B16 cells with IC_{50} values of $0.05\% \pm 0.007$ (24h), $0.04\% \pm 0.002$ (48h) and $0.04\% \pm 0.001$ (72h) (Table 2). Prolonged exposure time of 0.02% TTO from 24h to 48h significantly ($P < 0.05$) reduced B16 cellular viability from 90% to 71% (Figure 2). Concentrations of TTO (0.04-0.1%) after 24h, (0.01-0.1%) after 48h and (0.02-0.1%) after 72h all significantly ($P < 0.05$) reduced B16 cell viability (Figure 2).

TTO was least efficacious against the normal human fibroblast cells HF32, with IC_{50} values of $0.08\% \pm 0.01$ (24h), $0.07\% \pm 0.02$ (48h) and $0.07\% \pm 0.01$ (72h) (Table 2). Although there was a dose-dependent decrease in cellular viability, prolonged exposure time from 24h to 48h to 72h had no significant effect on the cells. Only concentrations of TTO (0.1%) after 24h, 0.06%-0.1% after 48h and (0.08-0.1%) after 72h significantly ($P < 0.05$) reduced HF32 cell viability (Figure 3)

Specifically, at all time points, 24h, 48h, and 72h, 0.04% TTO significantly ($P < 0.05$) reduced cell viability of AE17 cells to 37.24%, 10.77% and 5.63% (Figure 1) respectively; of B16 cells to 58.59%, 21.25% and 24.14% (Figure 2) respectively; compared with cell viability of HF32 cells after 24h, 48h and 72h of 84.86%, 72.99% and 87% (Figure 3) with the same concentration of TTO.

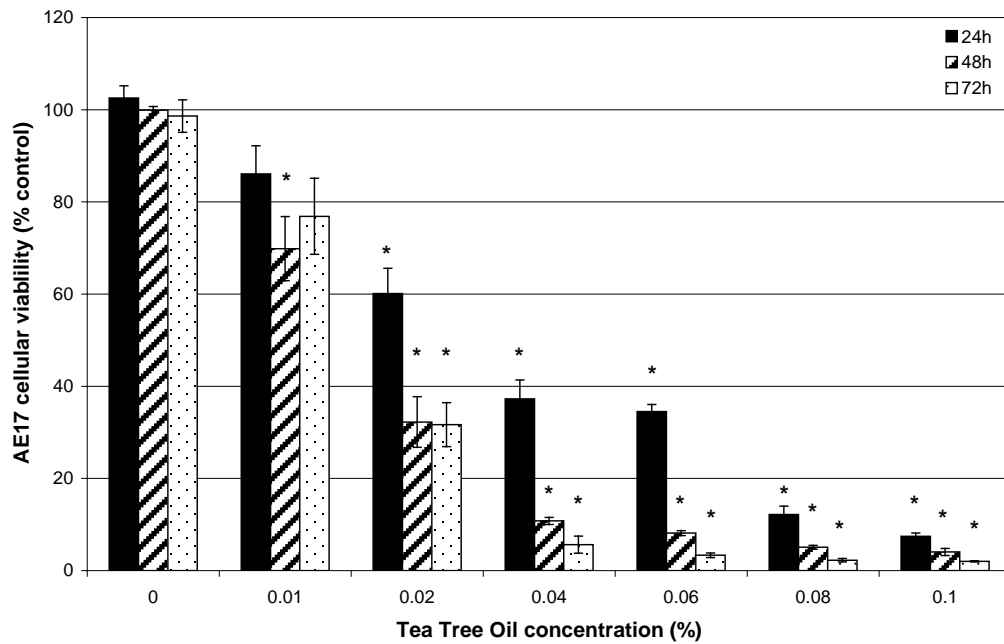


Figure 1: Effect of TTO on AE17 Mesothelioma cellular viability after 24-72h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean±S.E.M of at least 3 experiments. * indicates statistically significant ($P<0.05$) difference between solvent control and treatments.

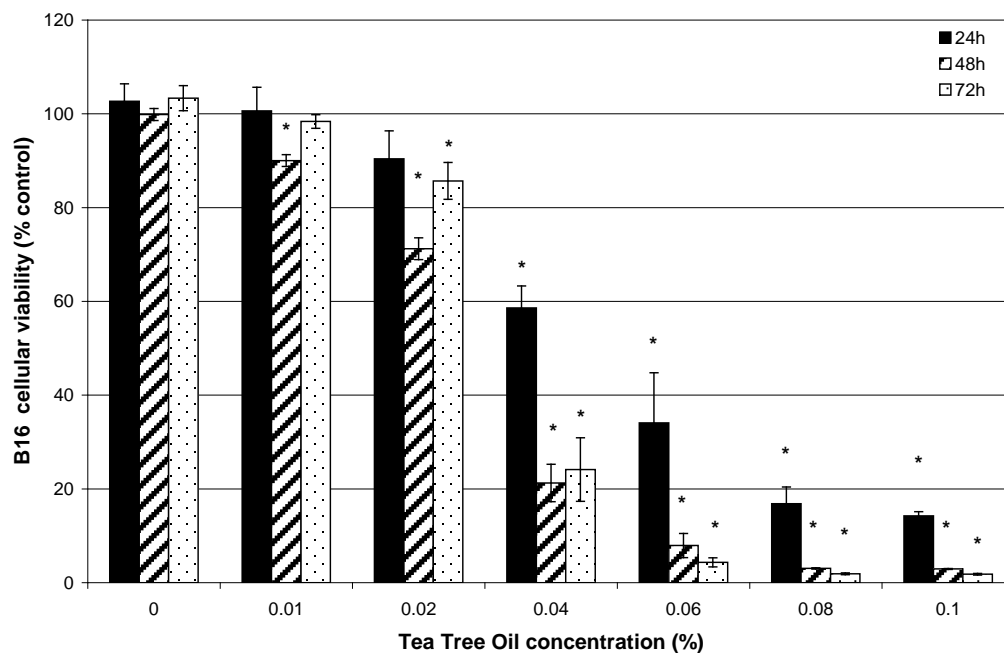


Figure 2: Effect of TTO on B16 Melanoma cellular viability after 24-72h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean±S.E.M of at least 3 experiments. * indicates statistically significant ($P<0.05$) difference between solvent control and treatments.

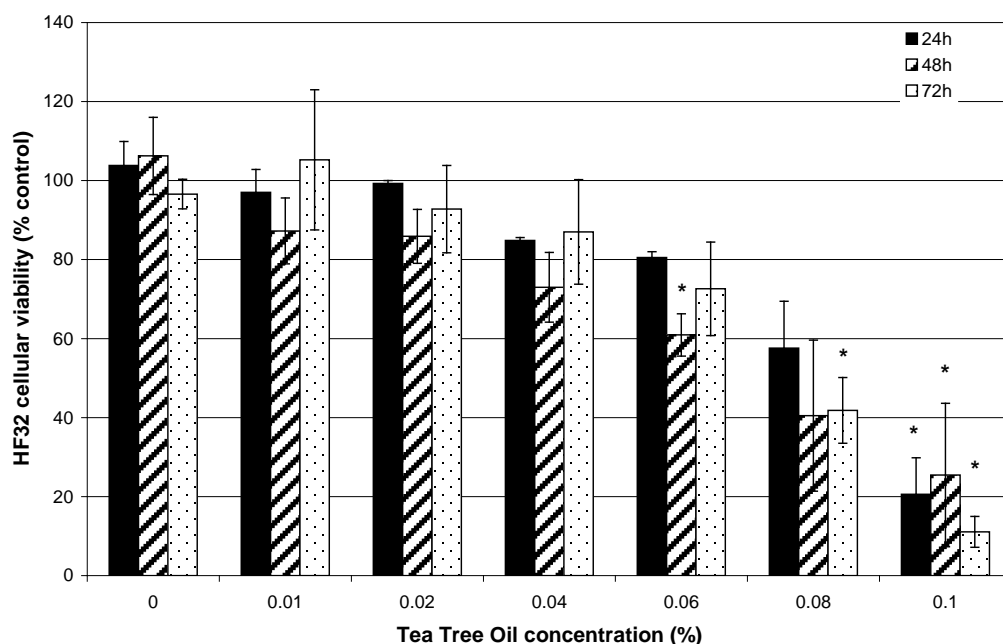


Figure 3: Effect of TTO on HF32 Fibroblast cellular viability after 24-72h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean \pm S.E.M of at least 2 experiments. * indicates statistically significant ($P<0.05$) difference between solvent control and treatments.

Terpinen-4-ol inhibits cellular viability

Terpinen-4-ol inhibited cellular viability of the two tumour cell lines studied AE17 Mesothelioma and B16 Melanoma, in a dose-and time-dependent manner as measured by the MTT assay (Table 2 and Figures 4 and 5).

Terpinen-4-ol was most efficacious against the AE17 cell line with IC_{50} values of $0.02\% \pm 0.001$ (24h), $0.01\% \pm 0.002$ (48h) and $0.02\% \pm 0.004$ (72h). Increasing exposure time from 24 to 48h significantly ($P<0.05$) decreased AE17 cellular viability (Table 2), with concentrations as low as 0.01% terpinen-4-ol (after 48h) significantly reducing viability to 50% compared with control cells (Figure 4). All concentrations (0.01-0.1%) of terpinen-4-ol after all exposure times (24-72h) significantly ($P<0.05$) reduced AE17 cell viability (Figure 4).

Terpinen-4-ol significantly reduced viability of B16 cells with IC_{50} values of $0.05\% \pm 0.009$ (24h), $0.04\% \pm 0.003$ (48h) and $0.02\% \pm 0.001$ (72h) (Table 2). Prolonged exposure time from 24h to 48h significantly ($P<0.05$) decreased B16 cellular viability (Table 2), with concentrations of terpinen-4-ol (0.04-0.1%) after 24h, (0.02-0.1%) after 48h and (0.06-0.1%) after 72h all significantly ($P<0.05$) reducing B16 cell viability (Figure 5).

Terpinen-4-ol was least efficacious against the normal human fibroblast cells HF32, with IC_{50} values of $0.13\% \pm 0.03$ (24h), $0.11\% \pm 0.03$ (48h) and $0.1\% \pm 0.03$ (72h) (Table 2). Only after 24h were concentrations of 0.08% and 0.1% significantly ($P<0.05$) cytotoxic to HF32 cells (Figure 6).

Specifically, at all time points, 24h, 48h, and 72h, 0.04% terpinen-4-ol significantly ($P<0.05$) reduced cell viability of AE17 cells to 37.1%, 7.4% and 8.6% (Figure 4) respectively; of B16 cells to 55.9%, 40.9% and 41.7% (Figure 5) respectively; compared with cell viability of HF32 cells after 24h, 48h and 72h of 83.85%, 78.22% and 78.6 % (Figure 6) with the same concentration of terpinen-4-ol.

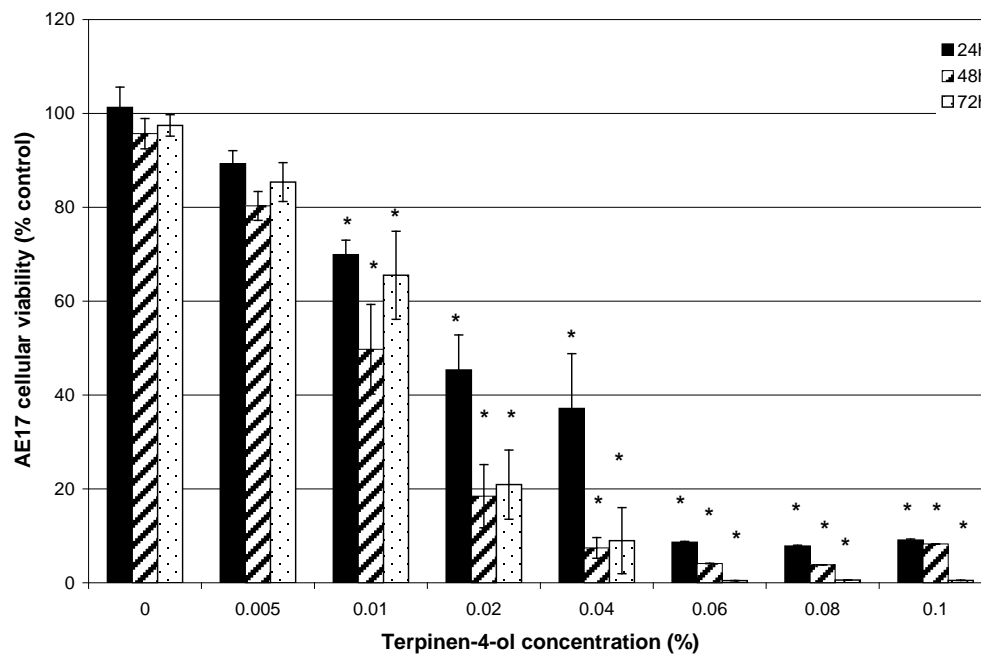


Figure 4: Effect of terpinen-4-ol on AE17 Mesothelioma cellular viability after 24-72h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean±S.E.M of at least 3 experiments. * indicates statistically significant ($P<0.05$) difference between solvent control and treatments

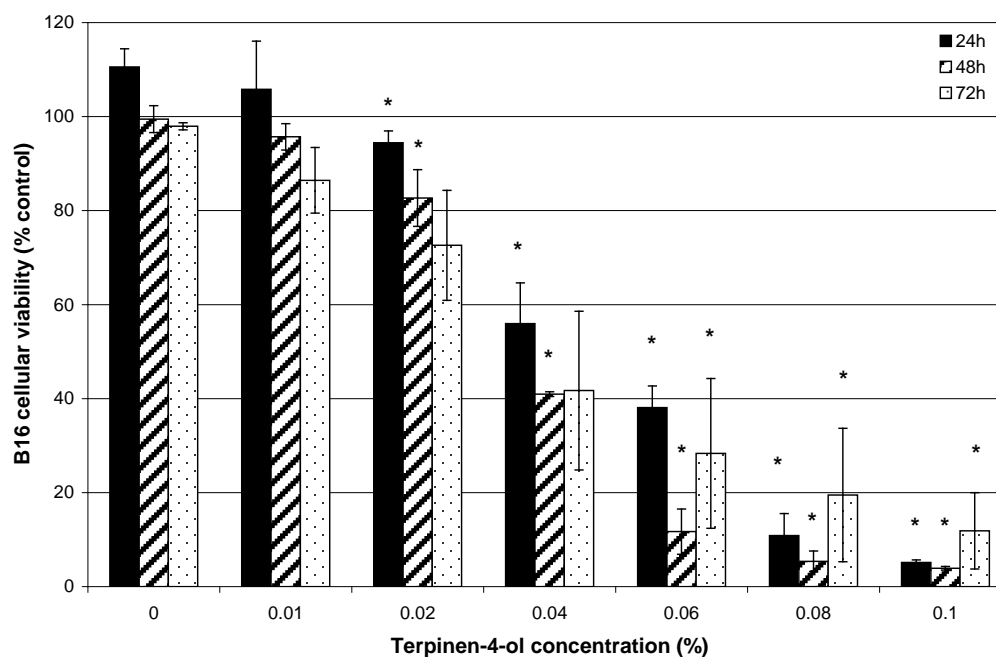


Figure 5: Effect of terpinen-4-ol on B16 Melanoma cellular viability after 24-72h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean±S.E.M of at least 3 experiments. * indicates statistically significant ($P<0.05$) difference between controls and treatments.

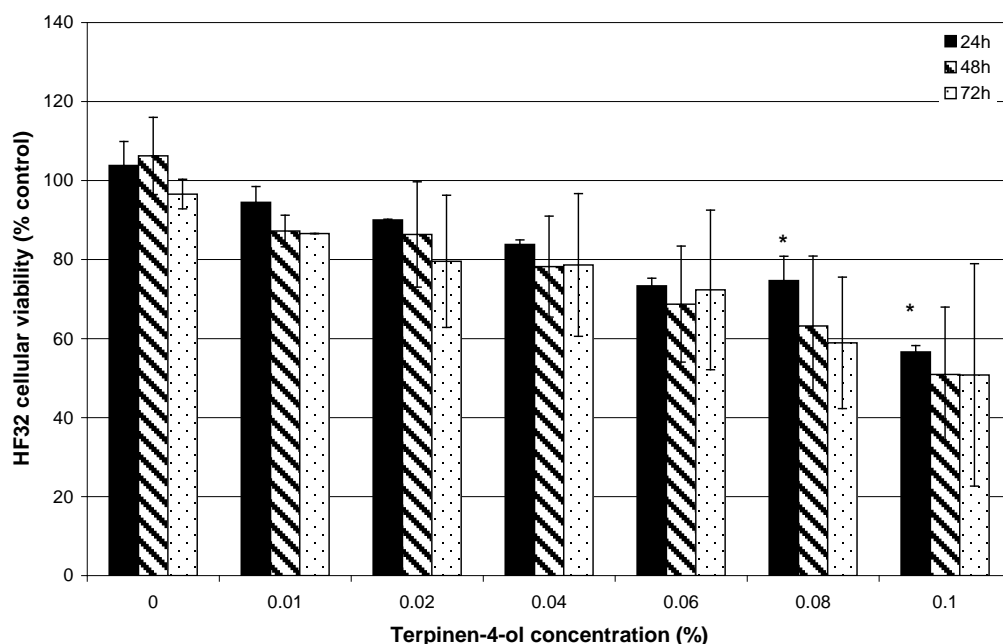


Figure 6: Effect of terpinen-4-ol HF32 Fibroblast cellular viability after 24-72h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean \pm S.E.M of at least 2 experiments. * indicates statistically significant ($P<0.05$) difference between controls and treatments.

“Mock” Tea tree oil inhibits cellular viability

“Mock” TTO inhibited AE17 and B16 cellular viability in a dose- and time-dependent manner (Figures 7 and 8) and was more efficacious against the AE17 cell line with IC_{50} values of $0.04\% \pm 0.01$ (24h), $0.02\% \pm 0.009$ (48h) and $0.03\% \pm 0.009$ (72h) and compared with B16 cell line with IC_{50} values of $0.08\% \pm 0.0004$ (24h), $0.06\% \pm 0.01$ (48h) and $0.06\% \pm 0.02$ (72h) (Table 2).

Specifically, at all exposure times, IC_{50} values of “Mock” TTO were not significantly different compared with TTO or terpinen-4-ol of AE17 cells (Table 2). After 24h the IC_{50} value of “Mock” TTO was significantly higher than that of TTO and terpinen-4-ol compared with B16 cells, but with prolonged exposure time, no significant difference between “Mock” TTO and TTO or terpinen-4-ol was evident.

Consistently, HF32 cells were significantly ($P<0.05$) least affected compared with the two tumour cell lines (Figure 9), with IC_{50} values of $0.15\% \pm 0.01$ (24h), $0.11\% \pm 0.008$ (48h) and $0.1\% \pm 0.01$ (72h) (Table 2). After 24h the IC_{50} value of “Mock” TTO (0.15) was significantly higher than that of TTO (0.08%), but with prolonged exposure time, no significant difference between “Mock” TTO and TTO or terpinen-4-ol was evident.

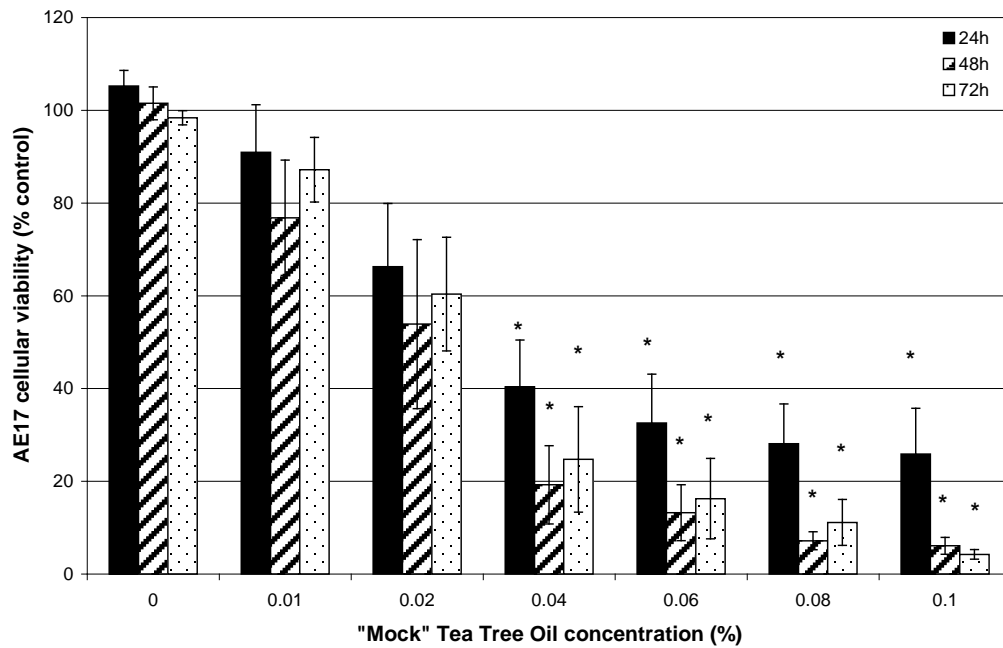


Figure 7: Effect of "Mock Tea Tree Oil" on AE17 Mesothelioma cellular viability after 24-72h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean±S.E.M of at least 2 experiments. * indicates statistically significant (P<0.05) difference between solvent control and treatments.

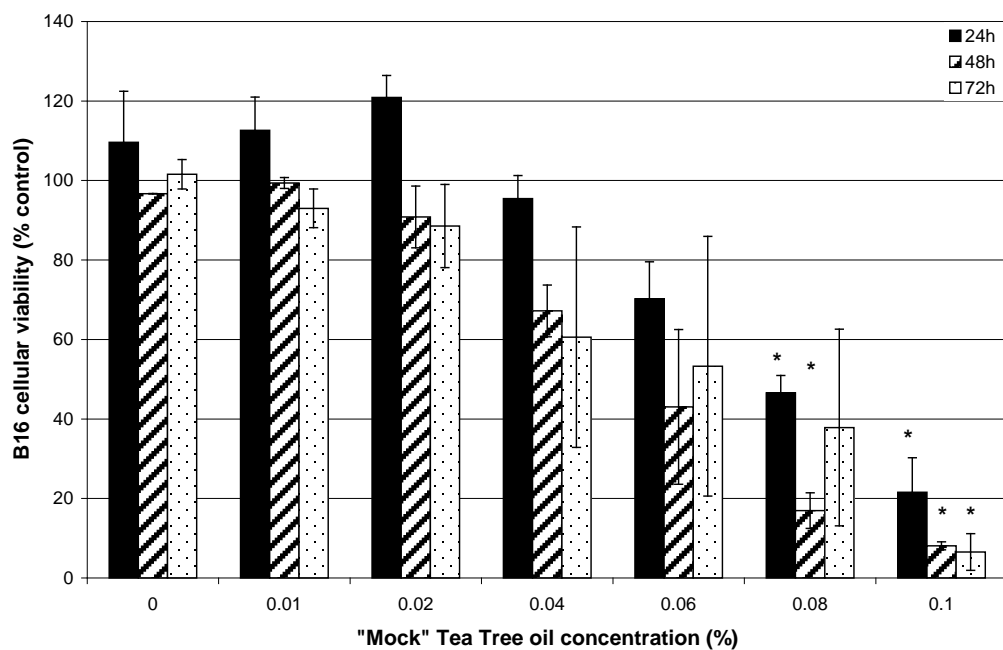


Figure 8: Effect of "Mock Tea Tree Oil" on B16 Melanoma cellular viability after 24-72h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean±S.E.M of at least 2 experiments. * indicates statistically significant (P<0.05) difference between solvent control and treatments.

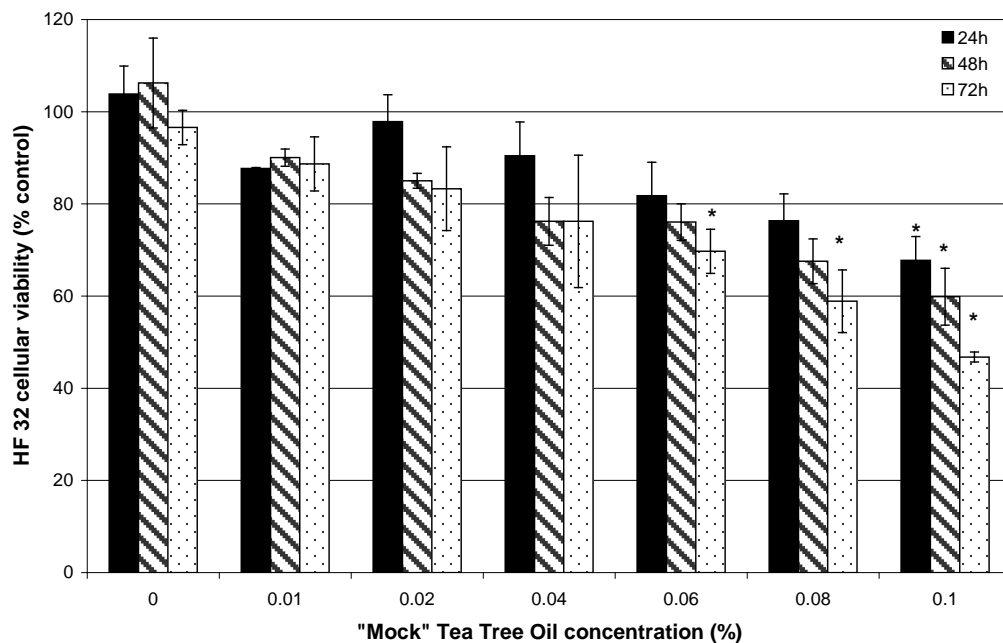


Figure 9: Effect of “Mock Tea Tree Oil” HF32 Fibroblast cellular viability after 24-72h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean±S.E.M of at least 2 experiments. * indicates statistically significant (P<0.05) difference between solvent control and treatments.

Table 2: ^a IC₅₀ values (%) for Tea Tree oil, terpinen-4-ol and “Mock” Tea Tree Oil after 24-72h against malignant mesothelioma (AE17), malignant melanoma (B16) and human fibroblast cells (HF32)

Cell Line	Tea Tree Oil			Terpinen-4-ol			“Mock” Tea Tree Oil		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
AE17	0.03±	0.02±	0.02±	0.02±	0.01±	0.02±	0.04±	0.02±	0.03±
	0.005	0.006	0.003	0.001	0.002	0.004	0.01	0.009	0.009
B16	0.05±	0.03±	0.03±	0.05±	0.04±	0.05±	0.08±	0.06±	0.06±
	0.007	0.002	0.001	0.009	0.003	0.001	0.001	0.01	0.02
HF32	0.08±	0.07±	0.07	0.13±	0.11	0.1±	0.15	0.11±	0.1±
	0.01	0.02	±0.01	0.03	±0.03	0.03	±0.01	0.008	0.01

^a IC₅₀ (%) is the amount of treatment required to inhibit cell growth by 50% compared to control cells and is calculated by linear/polynomial regression.

Data are the mean ±S.D of 6 replicates of at least 2 independent experiments

α - and γ -terpinene, 1,8-cineole and p-cymene do not inhibit cellular viability

The four other major components of TTO; α - and γ -terpinene, 1,8-cineole and p-cymene did not have a significant effect on any of the cell lines tested (Figures 10, 11 and 12) after 48h as assessed by the MTT assay.

At concentrations of 0.1% of all components tested; viability of AE17 cells did not decrease below 70% (Figure 10); viability of B16 cells did not decrease below 77% (Figure 11) and viability of HF32 cells did not decrease below 71% (Figure 12).

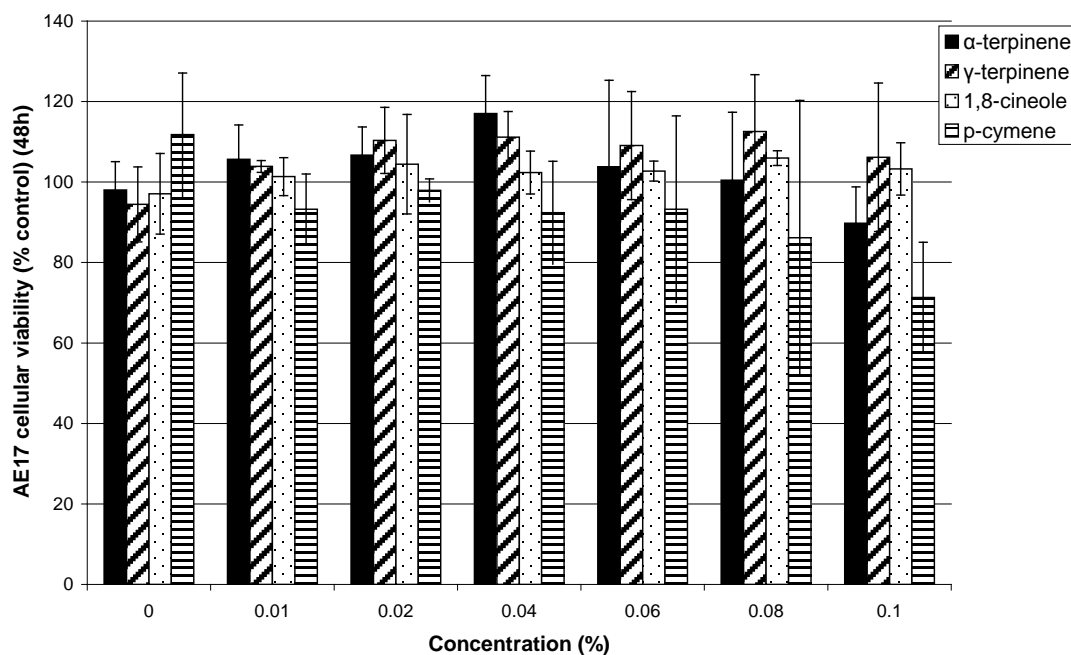


Figure 10: Effect of α - and γ -terpinene, 1,8-cineole and p-cymene on AE17 Mesothelioma cellular viability after 48h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean \pm S.E.M of 2 experiments. * indicates statistically significant ($P < 0.05$) difference between controls and treatments.

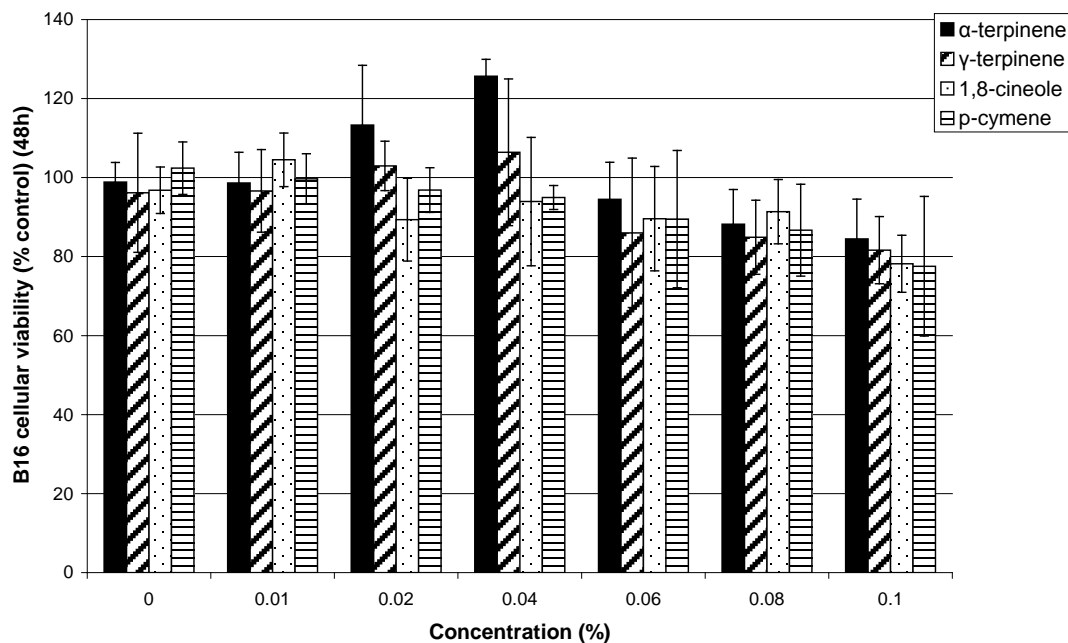


Figure 11: Effect of α - and γ -terpinene, 1,8-cineole and p-cymene on B16 Melanoma cellular viability after 48h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean \pm S.E.M of 2 experiments. * indicates statistically significant ($P<0.05$) difference between solvent controls and treatments.

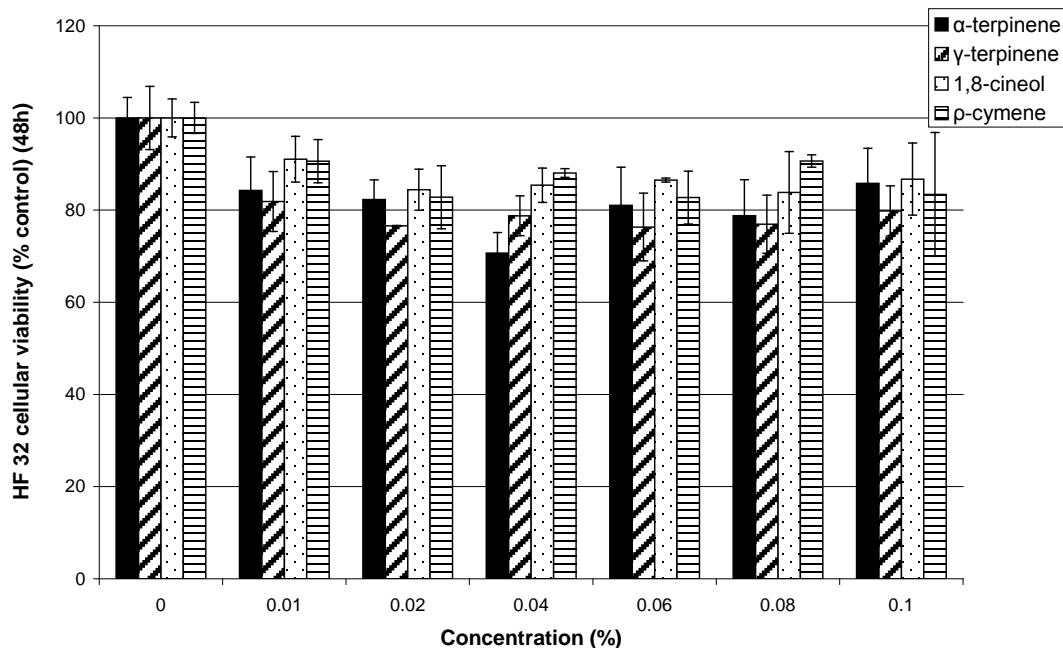


Figure 12: Effect of α - and γ -terpinene, 1,8-cineole and p-cymene on HF32 Fibroblast cellular viability after 48h as assessed by the MTT assay.

Results are expressed as % of control (DMSO 0.2% RPMI) and represent the mean \pm S.E.M of 2 experiments. * indicates statistically significant ($P<0.05$) difference between controls and treatments.

TTO and Terpinen-4-ol induce cell death by necrosis and apoptosis

Treatment of AE17 and B16 cells with TTO and terpinen-4-ol induced significant necrosis and apoptosis.

The morphological appearance of AE17 and B16 cells following treatment with TTO (0.02% and 0.04%) and terpinen-4-ol (0.02% and 0.04%) is depicted in Figures 13 and 14. After 48h exposure with TTO, some AE17 cells display brightly fluorescing Hoechst stained cells indicating apoptosis, displaying: cell shrinkage, membrane blebbing, nuclear condensation; with some bound Annexin-V-FITC (green) positive cells (which has a high affinity for phospholipid phosphatidylserine (PS) which is only exposed to the outer leaflet of the plasma membrane in early apoptotic cells), amongst a higher population of necrotic cells (Figure 13).

The primary mode of cell death appears to be necrosis, with characteristics including cell swelling and membrane rupture; this is confirmed by the uptake of propidium iodide (PI) (red cells); which only enters cells with compromised cell membranes. Staurosporine was included as a positive control which induced apoptosis: brightly fluorescing Hoechst stained cells, displaying cell shrinkage and blebbing, and bind Annexin-V-FITC (green cells) and necrosis with positively stained red PI cells (Figures 13 and 14). After 48h 0.04% TTO exposure (Figure 14), B16 cells have low level brightly fluorescing Hoechst stained cells and a lack of Annexin-V-FITC positive cells but are positive for necrotic cells. Concentrations of 0.02% and 0.04% terpinen-4-ol appear to induce cell death primarily by necrosis in both cell lines indicated by positive PI stained cells with a lack of Annexin-V-FITC positive cells (Figures 13 and 14).

In order to confirm this and quantify levels of apoptosis and necrosis following exposure to TTO and terpinen-4-ol, FACS was performed on double stained Annexin-V-PE and PI cell populations.

Specifically, 0.02% TTO elicited 12.5% necrosis and 5% apoptosis in AE17 cells. After 24h, increased exposure time did not significantly increase either type of cell death (15.3% necrosis and 5.5% apoptosis after 48h) (Figure 15). At a higher concentration of 0.04% TTO induced significantly more necrosis and apoptosis in AE17 cells; 36.2% necrosis, 13.3% apoptosis after 24h. Increased exposure time to 48h, induced significant higher level necrosis (48%) but apoptosis was similar (12.7%) (Figure 16).

0.04% TTO induced low levels of apoptosis and necrosis at both exposure times in B16 cells; 5.7% necrosis and 4.3% apoptosis after 24h (Figure 17) and 4.1% necrosis and 3.5% apoptosis after 48h (Figure 18).

After 24h, 0.06% TTO induced negligible necrosis and apoptosis in B16 cells (3.2% and 2.8% respectively) (Figure 17) but with an increased exposure time to 48h, 0.06% TTO induced significant ($P<0.05$) necrosis (17.4%) but again with little apoptosis (3.7%) (Figure 18).

Treatment with 0.02% terpinen-4-ol induced negligible necrosis and apoptosis at both exposure times (3% necrosis, 3% apoptosis 24, 48h) (Figures 15 and 16) in AE17 cells. However, with a higher concentration of 0.04%, terpinen-4-ol induced significant levels of both necrosis and apoptosis; 15.7% necrosis, 7.5% apoptosis after 24h. Increased exposure time to 48h, significantly increased necrosis (34.6%) but apoptosis levels were again similar (10.5%) (Figure 16).

Treatment with 0.04 and 0.06% terpinen-4-ol induced low levels of necrosis and apoptosis in B16 cells. Specifically, 0.04% elicited 8.8% necrosis, 3% apoptosis after 24h (Figure 17) and 1.3% necrosis and 6.1% apoptosis after 48h (Figure 18). Increased concentration to 0.06% induced 2.1% necrosis and 2.3% apoptosis after 24h and 10.6% necrosis and 5.4% apoptosis after 48h (Figures 17 and 18).

“Mock” TTO (0.04%) induced significant necrosis and apoptosis in AE17 cells at both exposure times; 32.6% necrosis and 10.5% apoptosis after 24h (Figure 15) and 17.9% necrosis and 10.3% apoptosis after 48h (Figure 16).

In B16 cells “Mock” TTO (0.04%) induced insignificant levels of necrosis and apoptosis after both exposure times; 4.7% necrosis and 3.8% apoptosis after 24h (Figure 17) and 2.5% necrosis and 3.6% apoptosis after 48h (Figure 18).

Staurosporine (10nM) included as a positive control induced 22.9% necrosis and 9.4% apoptosis in AE17 cells after 24h (Figure 15); with increased exposure time of 48h, staurosporine induced significant ($P<0.05$) 12.6% necrosis and 10.8% apoptosis (Figure 16). Staurosporine induced significant apoptosis in B16 cells at both exposure times; 11.6% after 24h and 9.6% after 48h but with negligible necrosis 1.1%, 1.2% after 24h (Figure 17) and 48h (Figure 18) respectively.

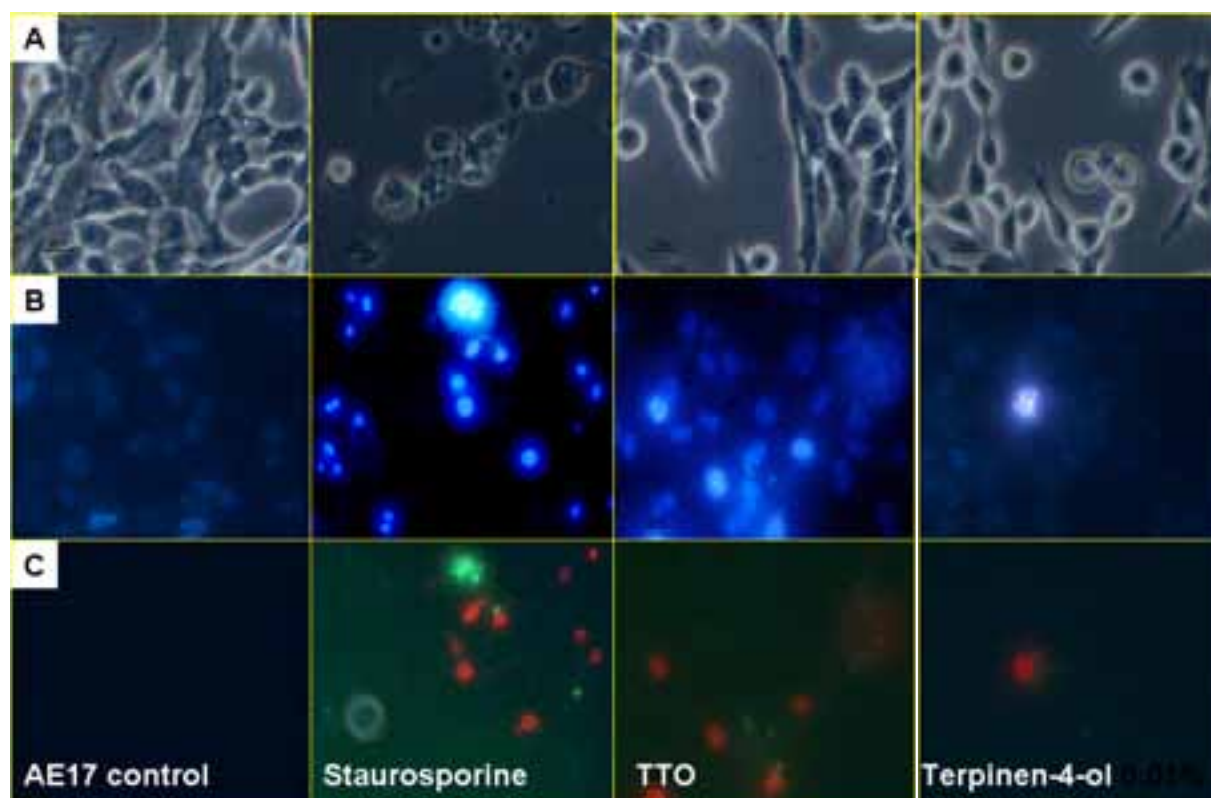
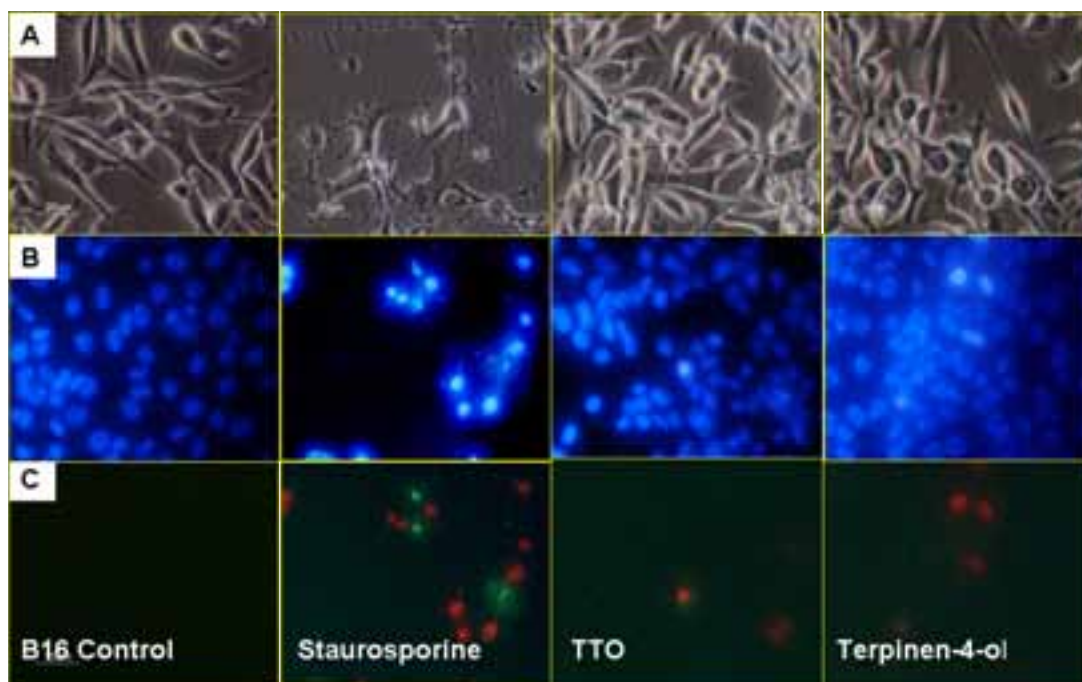


Figure 13: Phase contrast (A) and fluorescent (Hoechst (B) and annexin-V-FITC/propidium iodide (AV/PI) (C)) microscopy images (40x) of AE17 mesothelioma cells (48h) following; no treatment (Negative control); staurosporine (10nM) (Positive control); TTO 0.02%; terpinen-4-ol 0.02%.

Brightly fluorescing Hoechst cells indicate dead/dying cells; red florescence AV/PI indicate necrotic cells, green florescence AV/PI indicate apoptotic cells



**Figure 14: Phase contrast (A) and fluorescent (Hoechst (B) and Annexin-V-FITC/Propidium iodide (AV/PI) (C)) microscopy images (40x) of B16 Melanoma cells (48h) following; no treatment (Negative control); Staurosporine (10nM) (Positive control); TTO 0.04%; terpinen-4-ol 0.04%. **

Brightly fluorescing Hoechst cells indicate dead/dying cells; red florescence AV/PI indicate necrotic cells, green florescence AV/PI indicate apoptotic cells

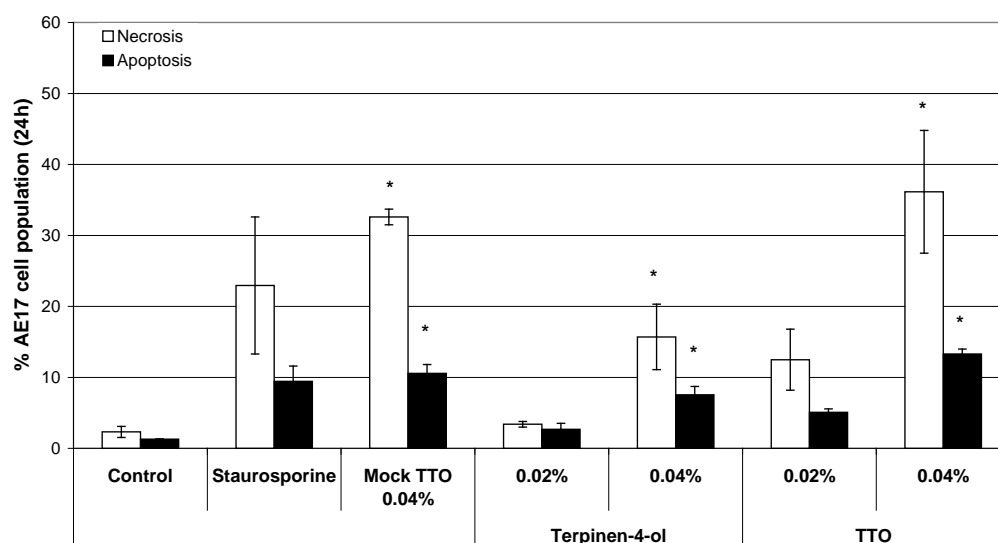


Figure 15: Effect of “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.02, 0.04%) and TTO (0.02, 0.04%) on apoptosis and necrosis of AE17 mesothelioma cells after 24h.

Apoptosis and necrosis were quantified by FACS using Annexin V-PE and 7AAD double staining. Untreated cells served as a negative control while Staurosporine (10nM) was included as a positive control. Data represent the mean \pm S.E.M of at least 2 independent experiments, quantifying a minimum of 10,000 cells per treatment. * indicates statistically significant ($P < 0.05$) difference in apoptosis or necrosis between control and treatments

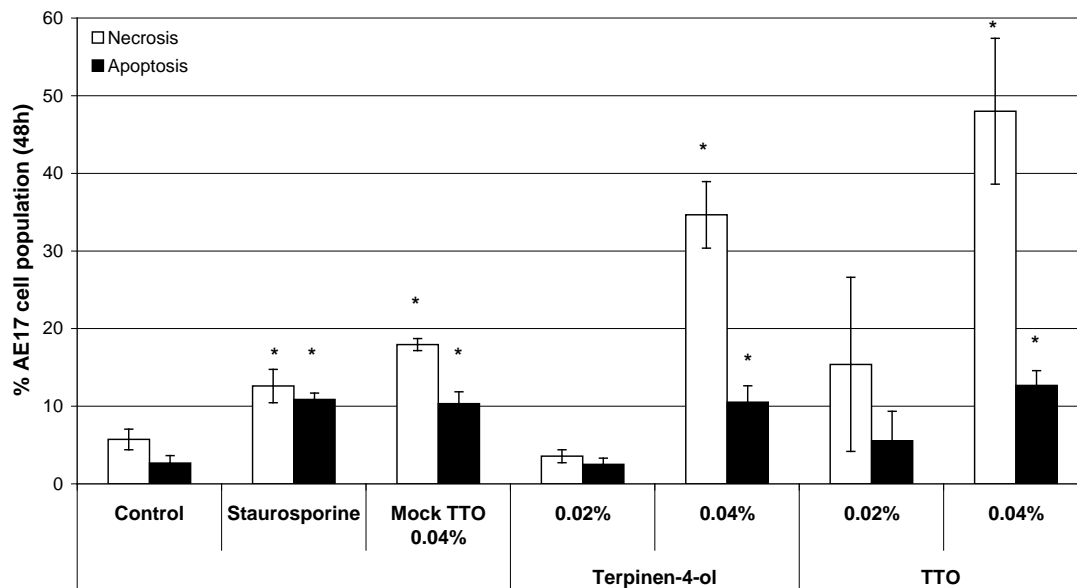


Figure 16: Effect of “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.02, 0.04%) and TTO (0.02, 0.04%) on apoptosis and necrosis of AE17 mesothelioma cells after 48h.

Apoptosis and necrosis were quantified by FACS using Annexin V-PE and 7AAD double staining. Untreated cells served as a negative control while Staurosporine (10nM) was included as a positive control. Data represent the mean \pm S.E.M of at least 2 independent experiments, quantifying a minimum of 10,000 cells per treatment.* indicates statistically significant ($P < 0.05$) difference in apoptosis or necrosis between control and treatments.

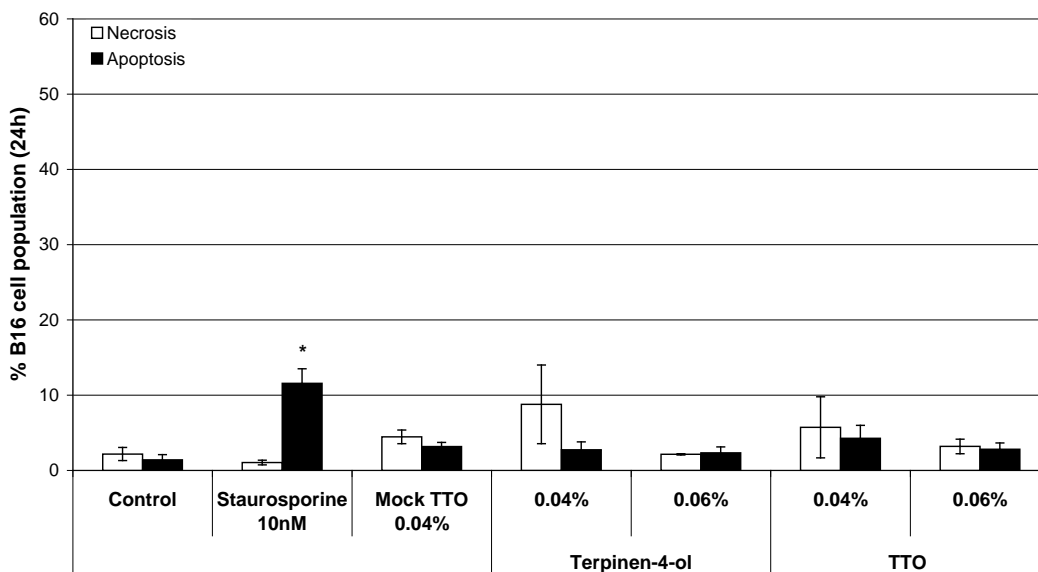


Figure 17: Effect of “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.04, 0.06%) and TTO (0.04, 0.06%) on apoptosis and necrosis of B16 melanoma cells after 24h.

Apoptosis and necrosis were quantified by FACS using Annexin V-PE and 7AAD double staining. Untreated cells served as a negative control while Staurosporine (10nM) was included as a positive control. Data represent the mean \pm S.E.M of at least 2 independent experiments, quantifying a minimum of 10,000 cells per treatment.* indicates statistically significant ($P < 0.05$) difference in apoptosis or necrosis between control and treatments.

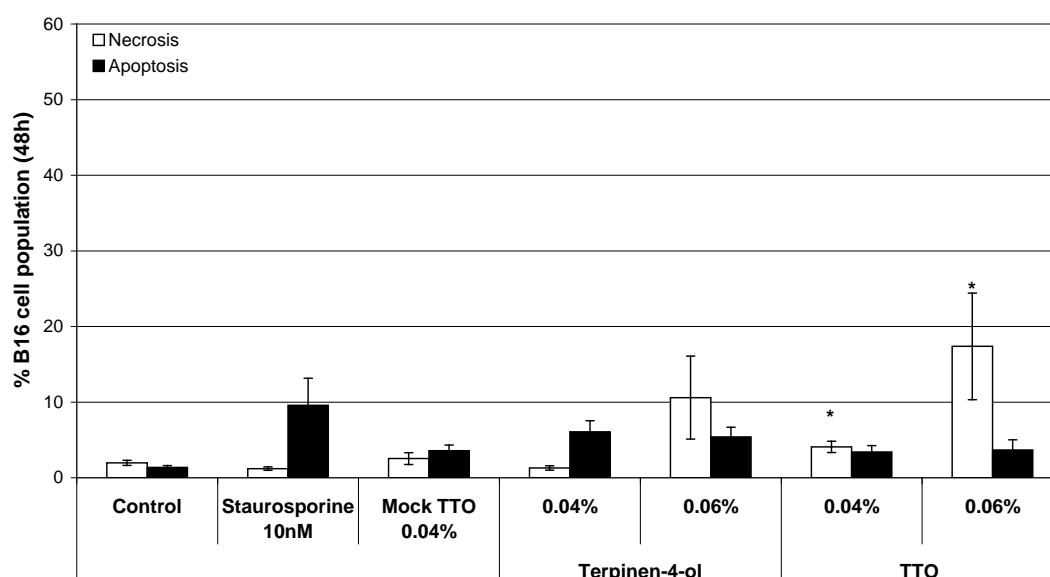


Figure 18: Effect of “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.04, 0.06%) and TTO (0.04, 0.06%) on apoptosis and necrosis of B16 melanoma cells after 24h.

Apoptosis and necrosis were quantified by FACS using Annexin V-PE and 7AAD double staining. Untreated cells served as a negative control while Staurosporine (10nM) was included as a positive control. Data represent the mean \pm S.E.M of at least 2 independent experiments, quantifying a minimum of 10,000 cells per treatment. * indicates statistically significant ($P < 0.05$) difference in apoptosis or necrosis between control and treatments.

TTO and terpinen-4-ol induce cell cycle arrest

Due to the low levels of apoptosis and levels of necrosis that did not explain cytotoxicity observed with the MTT assays; cell cycle distribution was analysed following treatment of AE17 and B16 cells with TTO and terpinen-4-ol over 12, 24 and 48h.

AE17 control cells had a cell cycle phase distribution of: 28% in G0/G1, 34% in S and 24.5% in G2/M after 12h (Figure 19). Following treatment with 0.04% terpinen-4-ol, cells in phase G0/G1 increased slightly ($P < 0.05$) by 4% (Figure 19). 10nM staurosporine significantly ($P < 0.05$) induced a G0/G1 arrest (increase by 16%) with a corresponding decrease (by 15%) in cells in S phase (Figure 19). No other treatments at this exposure time significantly induced cell cycle arrest.

After 24h, AE17 control cells had a cell cycle phase distribution of: 29% in G0/G1, 33.2% in S and 22.7% in G2/M (Figure 20). Following treatment with 0.02% and 0.04% TTO, AE17 cells displayed significant ($P < 0.05$) cell cycle arrest in G0/G1 (0.02% TTO increased G0/G1 by 5%, 0.04% TTO increased G0/G1 by 7%) with a corresponding significant decrease (by 5% and 7% respectively) in S phase (Figure 20). A similar effect was seen with 0.04% terpinen-4-ol; inducing G0/G1 cell cycle arrest (increase by 9%) with a corresponding decrease in S phase by 8% (Figure 20) 10nM staurosporine significantly ($P < 0.05$) induced a G0/G1 arrest (increase by 24%) with a corresponding decrease (by 19%) in cells in S phase (Figure 20).

After 48h, AE17 control cells had a cell cycle phase distribution of; 45.3% in G0/G1, 23.7% in S and 18.3% in G2/M (Figure 21). Staurosporine again induced a significant cell cycle arrest in G0/G1 (increase by 20%) and a decrease in S phase by 12%. At this higher exposure time, following treatment with 0.04% TTO, AE17 cells arrested in G2/M phase (increase by 8%) with a decrease in cells in G0/G1 (by 14%) (Figure 21).

After 12h and 48h “Mock” TTO had no significant effect on AE17 cell cycle distribution (Figures 19 and 21). After 24h a slight decrease in S phase by 5% was apparent, with a small increase in G2M by 3% (Figure 20).

After 12h, B16 control cells had a cell cycle phase distribution of: 33.9% in G0/G1, 41.4% in S and 13.8% in G2/M (Figure 22). Following treatment with TTO and terpinen-4-ol, B16 cells were significantly more affected compared with AE17 cells. Following treatment with 0.04% TTO, B16 cells displayed significant ($P<0.05$) cell cycle arrest in G0/G1 with an increase in G0/G1 by 24%, and a corresponding decrease by 23% in S phase. Following treatment with 0.06% TTO also induced cell cycle arrest in G0/G1 with an increase in G0/G1 by 23% with a corresponding significant decrease (by 25%) in S phase, but also induced cell cycle arrest in G2/M by increasing cells in this phase by 11% compared with control cells (Figure 22). A similar effect was seen with 0.04% terpinen-4-ol; inducing G0/G1 cell cycle arrest (increase by 30%) with a corresponding decrease in S phase by 25% (Figure 22), but with increased concentrations of terpinen-4-ol to 0.06%, a G0/G1 arrest was evident (increase by 20%, decrease in S by 25%) but also with B16 cell cycle arrest in G2/M (increase by 9%). 10nM staurosporine significantly ($P<0.05$) induced a G0/G1 arrest (increase by 24%) with a corresponding decrease (by 19%) in cells in S phase (Figure 22).

After 24h, B16 control cells had a cell cycle phase distribution of: 35.9% in G0/G1, 46% in S and 16% in G2/M (Figure 23). Following treatment with TTO and terpinen-4-ol B16 cells were again significantly more affected compared with AE17 cells. B16 cells treated with 0.04% TTO displayed significant ($P<0.05$) cell cycle arrest in G0/G1, with an increase in G0/G1 by 9%, and a corresponding decrease by 20% in S phase. Following treatment, 0.06% TTO also induced cell cycle arrest in G0/G1 with an increase in G0/G1 by 11% with a corresponding significant decrease (by 22%) in S phase, but also induced a slight cell cycle arrest in G2/M by increasing cells in this phase by 6% compared with control cells (Figure 23). A similar effect was seen with 0.04% terpinen-4-ol; inducing G0/G1 cell cycle arrest (increase by 17%) with a corresponding decrease in S phase by 22% (Figure 23), with increased concentrations of terpinen-4-ol to 0.06%, a G0/G1 arrest was evident (increase by 20%) and a decrease in S phase by 24%. 10nM staurosporine significantly ($P<0.05$) induced a G0/G1 arrest (increase by 20%) with a corresponding decrease (by 39%) in cells in S phase (Figure 23).

After 48h, B16 control cells had a cell cycle phase distribution of; 64% in G0/G1, 18.2% in S and 14.6% in G2/M (Figure 24). Staurosporine significantly reduced the number of B16 cells in G2/M (by 7%) (Figure 24). With the increased exposure time, treatment with 0.04% TTO significantly reduced the number of cells in G0/G1 (by 18%) and with an increase in concentration to 0.06%, TTO induced B16 cell cycle arrest in G2/M phase (increase by 5%) with a decrease in cells in G0/G1 (by 20%) (Figure 24). Again with the increased exposure time to 48h, terpinen-4-ol at concentrations of 0.04% and 0.06% decreased cells cycling in G0/G1 (by 9 and 13% respectively) (Figure 24).

After 12h “Mock” TTO (0.04%) induced B16 cell G0/G1 phase arrest (increase by 22%) and a corresponding decrease in S by 16% (Figure 22). With increased exposure time to 24h “Mock” TTO induced lower level G0/G1 arrest (increase by 7%) with a decrease in S phase by 16% (Figure 23). Following treatment for 48h with “Mock” TTO, there was no apparent effect on B16 cell cycle distribution (Figure 24).

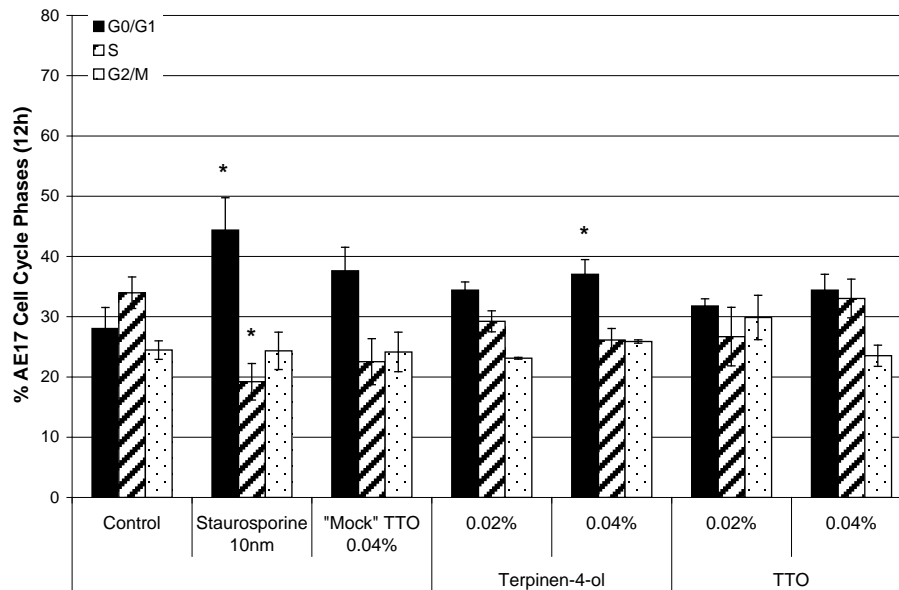


Figure 19: Cell cycle analysis by FACS of propidium iodide stained AE17 mesothelioma cells following treatment with “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.02, 0.04%) and TTO (0.02, 0.04%) after 12h.

Results are expressed as percentages of cell cycle distribution in phases: G0 /G1, S and G2/M calculated by DNA content analysis using the Dean-Jett-Fox model in FlowJo software and are compared with control, untreated cells; staurosporine (10nM) was included as a positive control. Data represent the mean \pm S.E.M of 3 independent experiments, quantifying a minimum of 10,000 events per treatment of only (2N/4N) gated cells. * indicates statistically significant ($P<0.05$) difference in cell cycle phase between control and treatments.

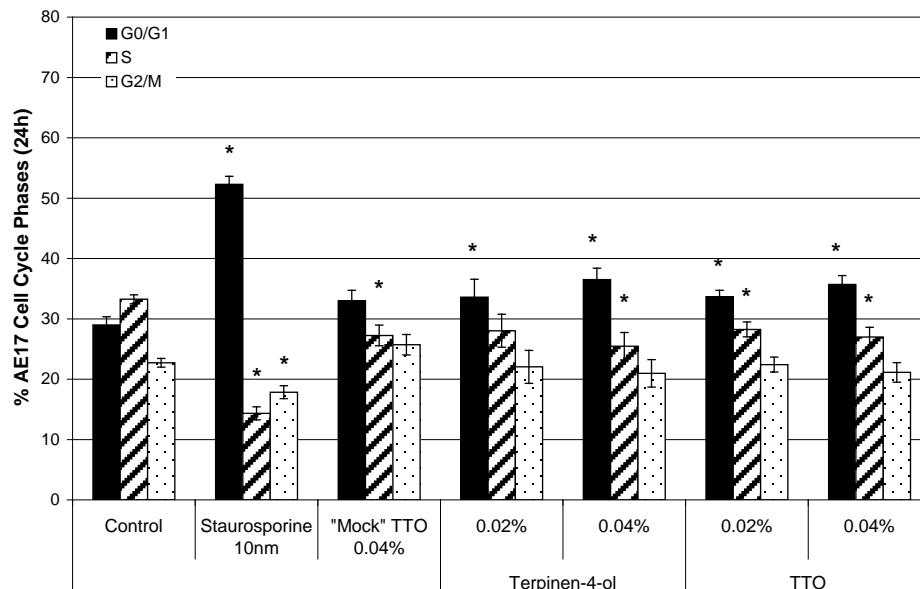


Figure 20: Cell cycle analysis by FACS of propidium iodide stained AE17 mesothelioma cells following treatment with “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.02, 0.04%) and TTO (0.02, 0.04%) after 24h.

Results are expressed as percentages of cell cycle distribution in phases: G0 /G1, S and G2/M calculated by DNA content analysis using the Dean-Jett-Fox model in FlowJo software and are compared with control, untreated cells; staurosporine (10nM) was included as a positive control.. Data represent the mean \pm S.E.M of 5 independent experiments, quantifying a minimum of 10,000 events per treatment of only (2N/4N) gated cells. * indicates statistically significant ($P<0.05$) difference in cell cycle phase between control and treatments.

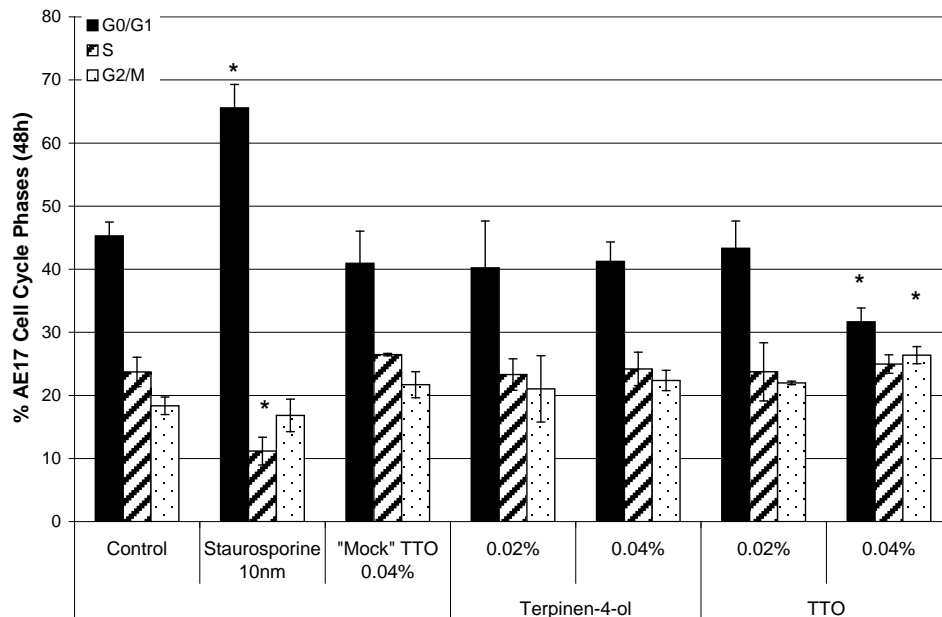


Figure 21: Cell cycle analysis by FACS of propidium iodide stained AE17 mesothelioma cells following treatment with “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.02, 0.04%) and TTO (0.02, 0.04%) after 48h.

Results are expressed as percentages of cell cycle distribution in phases: G0 /G1, S and G2/M calculated by DNA content analysis using the Dean-Jett-Fox model in FlowJo software and are compared with control, untreated cells; staurosporine (10nM) was included as a positive control.. Data represent the mean \pm S.E.M of 2 independent experiments, quantifying a minimum of 10,000 events per treatment of only (2N/4N) gated cells. * indicates statistically significant ($P < 0.05$) difference in cell cycle phase between control and treatments.

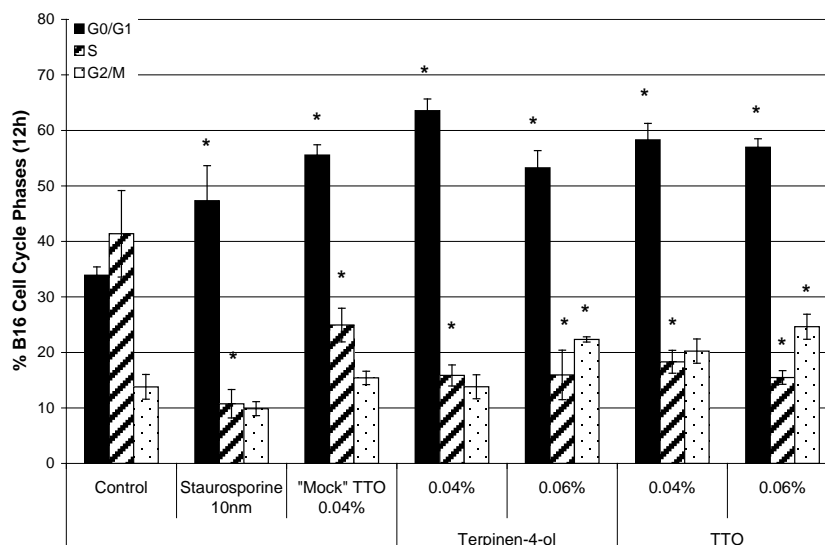


Figure 22: Cell cycle analysis by FACS of propidium iodide stained B16 melanoma cells following treatment with “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.04, 0.06%) and TTO (0.04, 0.06%) after 12h.

Results are expressed as percentages of cell cycle distribution in phases: G0 /G1, S and G2/M calculated by DNA content analysis using the Dean-Jett-Fox model in FlowJo software and are compared with control, untreated cells; staurosporine (10nM) was included as a positive control.. Data represent the mean \pm S.E.M of 3 independent experiments, quantifying a minimum of 10,000 events per treatment of only (2N/4N) gated cells. * indicates statistically significant ($P < 0.05$) difference in cell cycle phase between control and treatments.

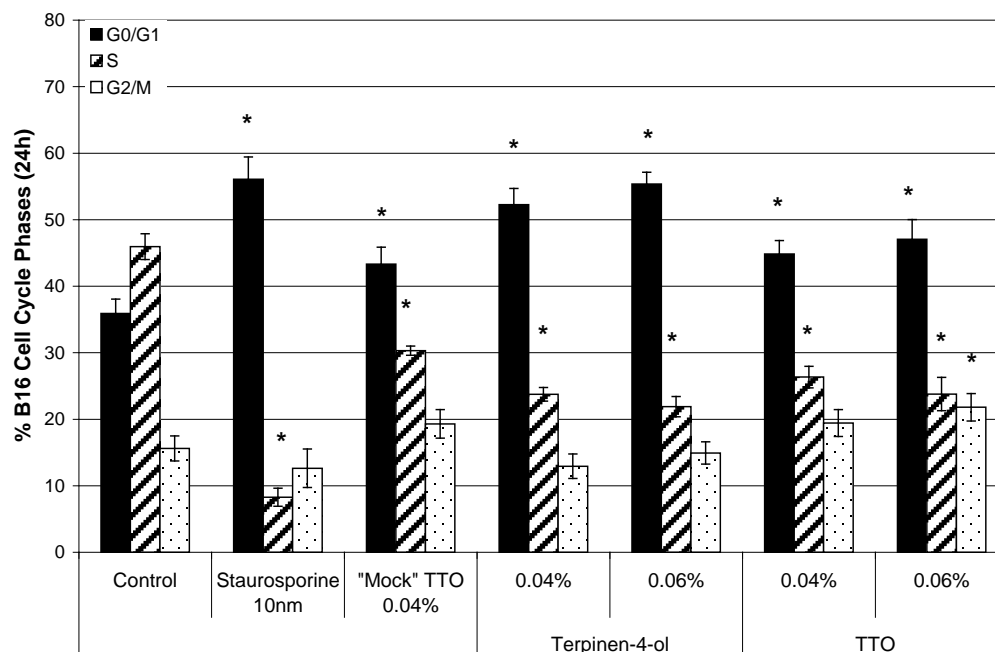


Figure 23: Cell cycle analysis by FACS of propidium iodide stained B16 melanoma cells following treatment with “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.04, 0.06%) and TTO (0.04, 0.06%) after 24h.

Results are expressed as percentages of cell cycle distribution in phases: G0 /G1, S and G2/M calculated by DNA content analysis using the Dean-Jett-Fox model in FlowJo software and are compared with control, untreated cells; staurosporine (10nM) was included as a positive control.. Data represent the mean \pm S.E.M of 5 independent experiments, quantifying a minimum of 10,000 events per treatment of only (2N/4N) gated cells. * indicates statistically significant ($P<0.05$) difference in cell cycle phase between control and treatments.

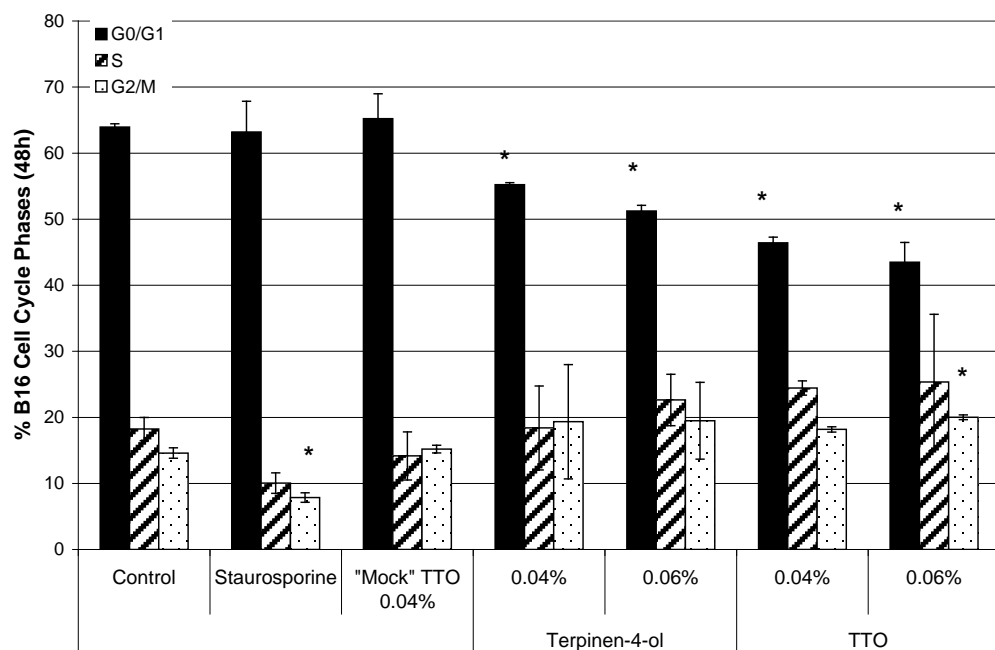


Figure 24: Cell cycle analysis by FACS of propidium iodide stained B16 melanoma cells following treatment with “Mock” Tea Tree oil (0.04%), terpinen-4-ol (0.04, 0.06%) and TTO (0.04, 0.06%) after 48h.

Results are expressed as percentages of cell cycle distribution in phases: G0 /G1, S and G2/M calculated by DNA content analysis using the Dean-Jett-Fox model in FlowJo software and are compared with control, untreated cells; staurosporine (10nM) was included as a positive control.. Data represent the mean \pm S.E.M of 2 independent experiments, quantifying a minimum of 10,000 events per treatment of only (2N/4N) gated cells. * indicates statistically significant ($P<0.05$) difference in cell cycle phase between control and treatments.

***In vivo* experiments**

10% TTO inhibits AE17 and B16 tumour growth and induces AE17 tumour regression

Mice with established (~9mm²) AE17 (Figure 25) and B16 (~15mm²) (Figure 26) implanted subcutaneous tumours, were treated topically with 50µl 10% TTO in DMSO or DMSO alone (solvent control) for 4 days.

TTO completely significantly ($P<0.05$) inhibited mesothelioma (AE17) tumour growth and significantly ($P<0.05$) reduced tumour area by ~100% during treatment (2 groups of 3 mice) (Figure 25). Following just one treatment of TTO in DMSO, tumour area was significantly reduced by 6-fold (Figure 25). Upon cessation of treatment tumours relapsed but with some degree of variability; ranging from 4-12 days post treatment, whilst 2 out of 9 of the mice displayed no signs of relapse until 3 months post treatment.

TTO significantly ($P<0.05$) retarded melanoma (B16) tumour growth by ~50% following 2 days post treatment (3 out of 3 mice) (Figure 26). Tumours did resume growth upon cessation of treatment in 2 of the 3 mice, and grew rapidly to 100mm² following 4 days post treatment. Side effects manifested as skin irritation; dryness, erythema, edema, with eschar formation but began to heal 3 days post cessation of treatment and were completely resolved by day 10.

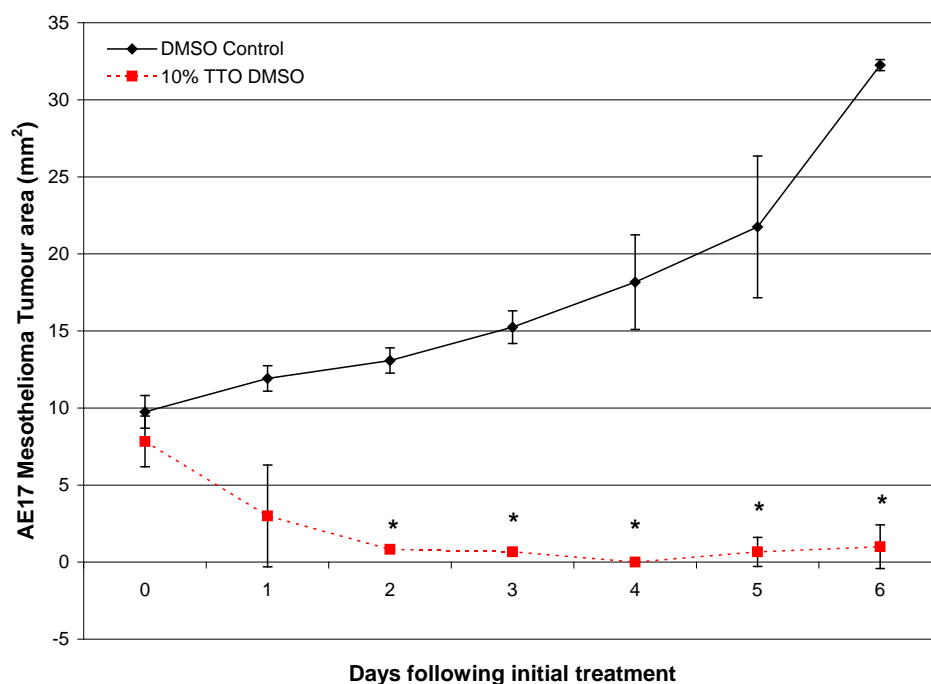


Figure 25: C57BL/6J mice with subcutaneous tumours (~9mm² AE17) treated topically daily for 4 days with 50µl DMSO alone (solvent control) or 50µl of 10% TTO in DMSO (n=3 for all groups).

Data are represented as the mean \pm S.E.M of 3 independent experiments (totalling 9 mice per treatment group). * indicate statistically significantly different ($P<0.05$) from control mice.

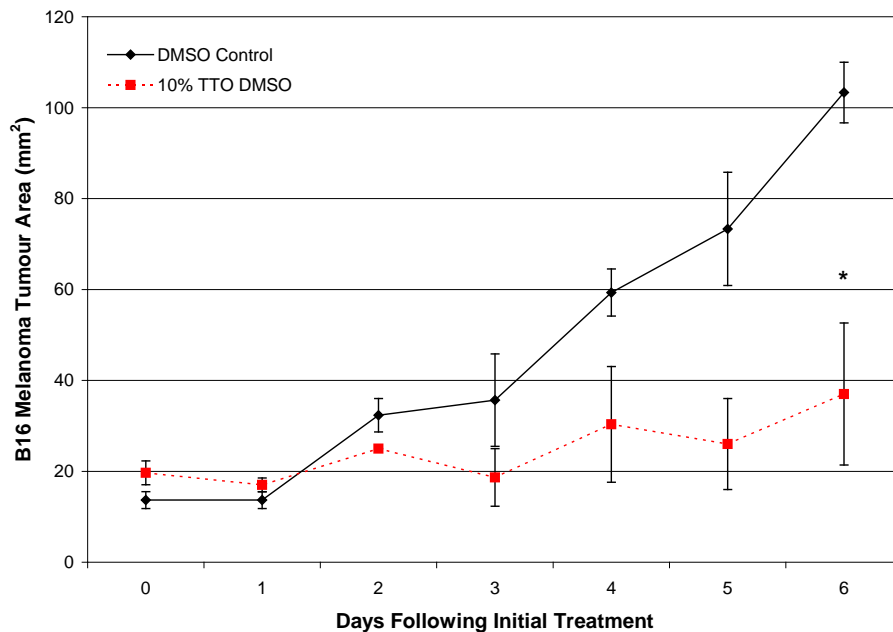


Figure 26: C57BL/6J mice with subcutaneous tumours (~15mm² B16) treated topically daily for 4 days with 50µl DMSO alone (solvent control) or 50µl of 10% TTO in DMSO (n=3 for all groups).

Data are represented as the mean \pm S.D of 1 independent experiment. * indicate statistically significantly different ($P < 0.05$) from control mice.

3% TTO inhibits AE17 and B16 tumour growth and induces AE17 tumour regression

Mice with established (~9mm²) AE17 (Figure 27) and non established B16 (~0mm²) (Figure 28) implanted subcutaneous tumours, were treated topically with 50µl 3% TTO in DMSO, 50µl 3% TTO Novasel® Gel or DMSO alone (solvent control) for 16 days (AE17) and 13 days (B16).

3% Novasel® TTO gel inhibited mesothelioma (AE17) tumour growth but did not induce tumour regression (Figure 27). No side effects were evident; skin remained normal. Upon cessation of treatment tumours resumed growth but were significantly ($P < 0.05$) slow growing with a tumour area of 9mm² compared to control tumours of 77 mm² at day 25 (Figure 27) (2 out of 3 mice).

3% TTO in DMSO completely inhibited mesothelioma (AE17) tumour growth and induced significant ($P < 0.05$) tumour regression following just one treatment (Figure 27). ~100% tumour regression was induced by day 4 (6 out of 6 mice) (Figure 27). Side effects manifested as some skin dryness but again healed completely following cessation of treatment. Tumour growth resumed in 5 out of 6 mice but by day 22 (6 days post treatment) were still significantly ($P < 0.05$) smaller (15mm²) than control mice (52mm²) mice (Figure 27). Tumour failed to relapse in 1 mouse and remained tumour free for 3 months.

3% Novasel® TTO gel and 3% TTO in DMSO also inhibited B16 melanoma tumour (Figure 28) growth but did not induce tumour regression, without any skin side effects following gel treatment but again with some skin dryness with TTO in DMSO treatment. Following 6 days of treatment, tumours began to growth; but remained significantly ($P < 0.05$) slower growing (8-16mm², 9 days post tumour inoculation) compared with control DMSO alone treated tumours which reached ~100mm² 9 days post tumour inoculation (treated tumours reached ~90mm² in 2/3 mice 14 days post tumour inoculation) (Figure 28).

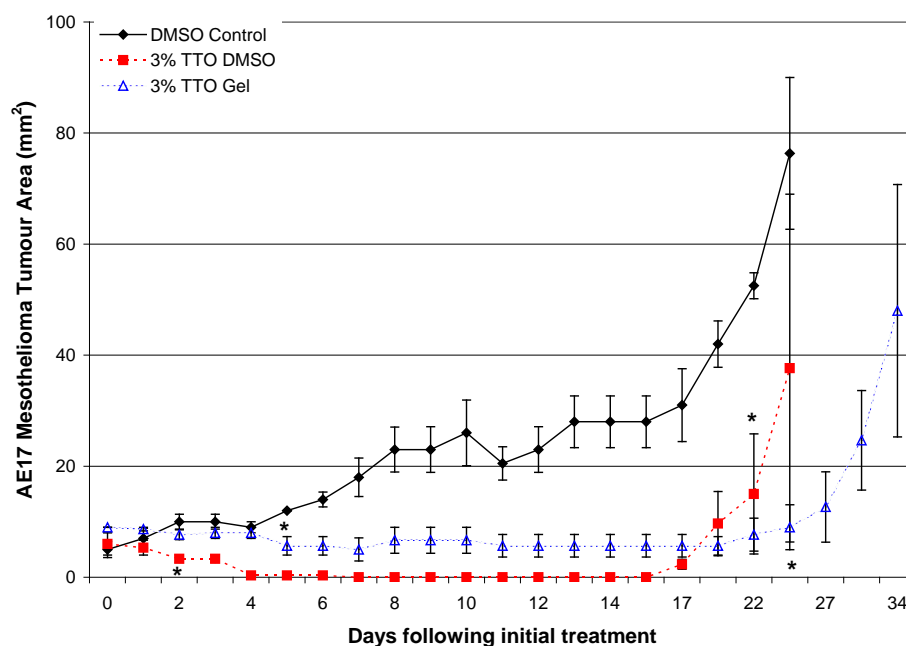


Figure 27: C57BL/6J mice with subcutaneous tumours (~9mm² AE17) treated topically daily for 16 days with 50µl DMSO (solvent control), 50µl 3% TTO in DMSO or 3% TTO Novasel® Gel (n=3 for all groups).

Data are represented as the mean \pm S.E.M of 2 independent experiments (totalling 6 mice per treatment group). * indicate statistically significantly different ($P < 0.05$) from control mice; all treatments of 3% TTO DMSO (day 2 to day 22) and all treatments of 3% TTO Novasel® Gel (day 5 to day 25)

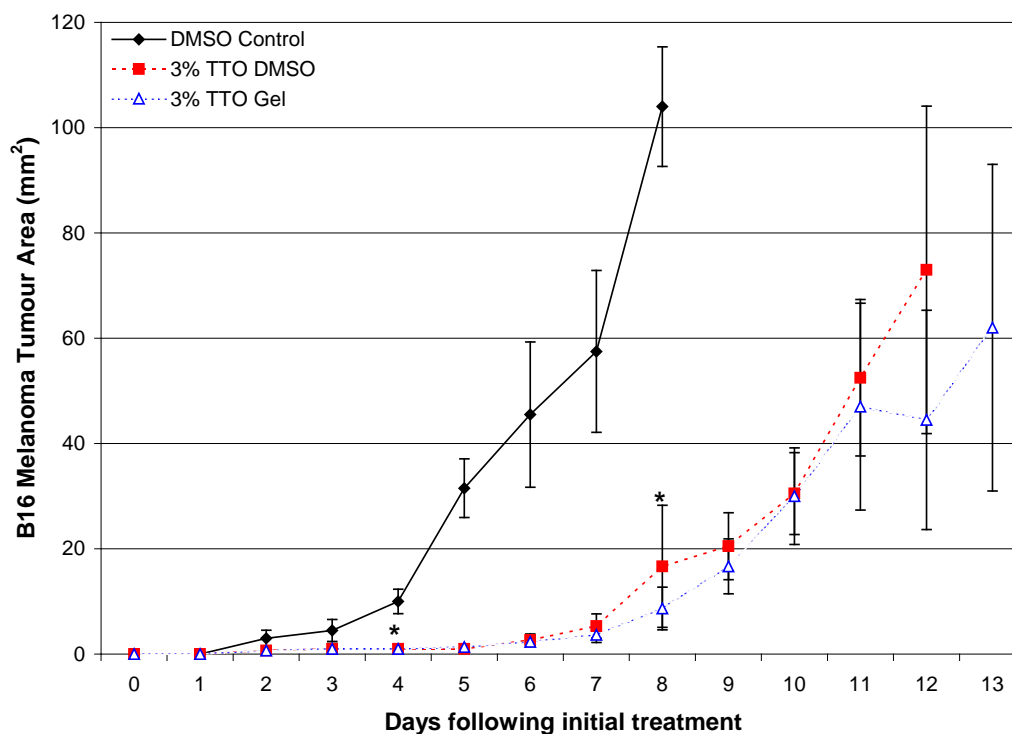


Figure 28: C57BL/6J mice with subcutaneous tumours (~0mm² B16, non-established) treated topically daily for 13 days with 50µl DMSO (solvent control), 50µl 3% TTO in DMSO or 3% TTO Novasel® Gel (n=3 for all groups).

Data are represented as the mean \pm S.D of 1 experiment. * indicate statistically significantly different ($P < 0.05$) from control mice; all treatments of 3% TTO DMSO and 3% and TTO Novasel® between day 4 and day 8.

5-15% TTO Novasel® Gel/Cream failed to inhibit B16 tumour growth or cause tumour regression

Mice with established $\sim 15\text{mm}^2$ B16 implanted subcutaneous tumours were treated topically with $\sim 50\mu\text{l}$ twice daily 5% TTO Novasel® Gel or 5% TTO Novasel® after-wax cream or 0% TTO Novasel® Gel (control) for 8 days.

At no time or treatment did either formulation inhibit B16 growth or induce tumour regression (Figure 29).

Mice with non-established $\sim 0\text{mm}^2$ but inoculated B16 implanted subcutaneous tumours were treated topically with $\sim 50\mu\text{l}$ daily with 0% Novasel® (control) gel, a 5%, 10% and 15% Novasel® TTO gel for 10 days.

At no time or treatment did any concentration of TTO gel inhibit B16 growth or induce tumour regression (Figure 30).

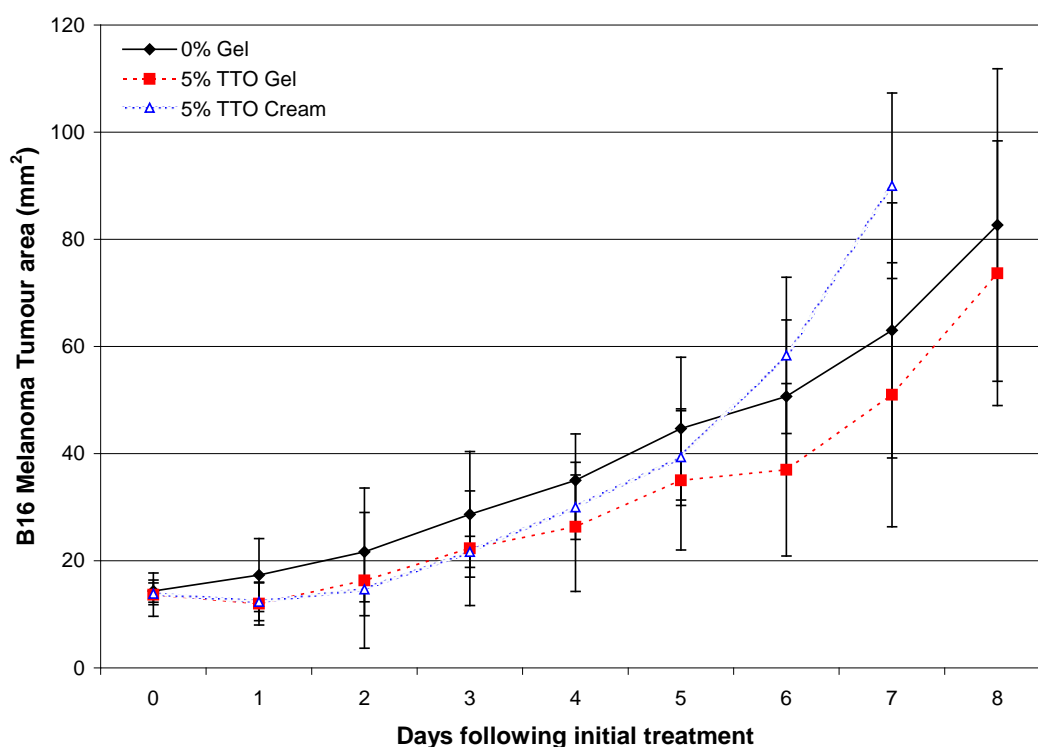


Figure 29: C57BL/6J mice with subcutaneous tumours ($\sim 15\text{mm}^2$ B16) treated topically twice daily for 8 days with: 0% Novasel® control gel, a 5% Novasel® TTO gel or a 5% Novasel® TTO after-wax cream (n=3 for all groups)

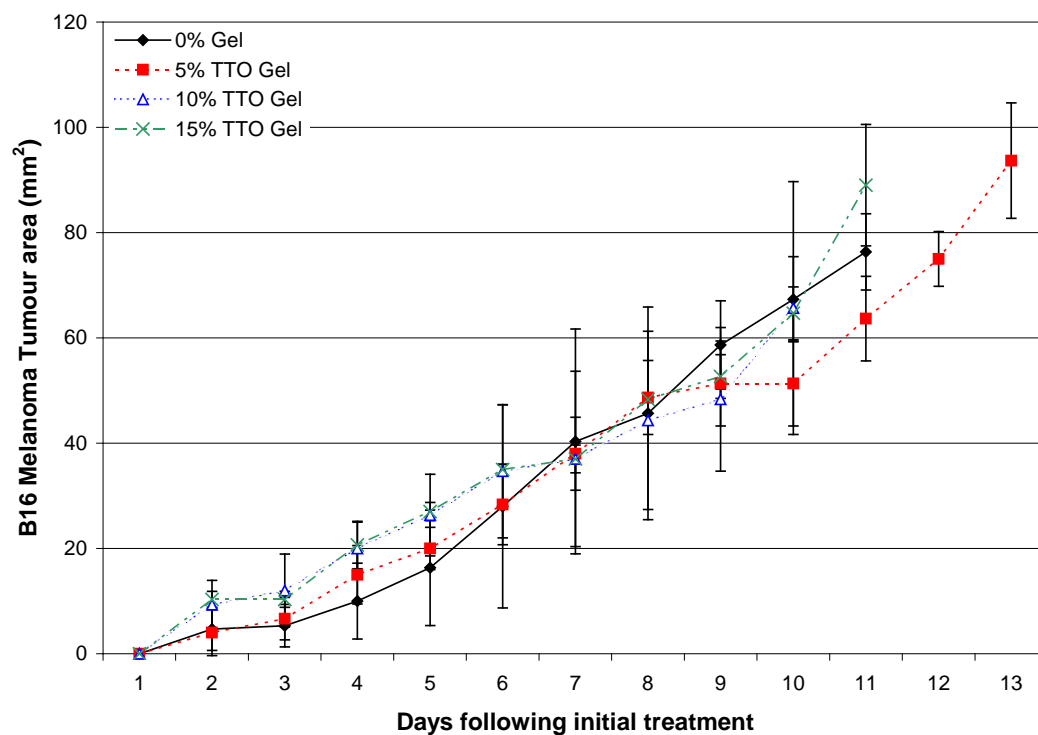


Figure 30: C57BL/6J mice with subcutaneous tumours (0mm² B16, non-established) treated topically daily for 10 days with: ~50µl 0% Novasel® control gel, a 5%, 10% and 15% Novasel® TTO gel (n=3 for all groups)

5. Discussion

In vitro experiments

Cytotoxicity of Tea tree oil and components

TTO significantly affects the viability of two murine tumour cell lines (AE17 and B16) in a dose- and time-dependent manner as assessed by the MTT assay. This effect is significantly pronounced in AE17 cells with equivalent concentrations of TTO reducing viability 1.5-1.6 fold more (24-72h) than in B16 melanoma cells. TTO had a dose-dependent effect against normal human fibroblasts HF32. However, this effect was only significantly cytotoxic at doses 3-fold greater (24-72h) compared with AE17 cells and 2-fold greater (24-72h) compared with B16 cells. This differential effect between tumour cells and normal cells is extremely encouraging. Previously reported IC_{50} value for TTO against fibroblasts after 24h of 0.067% (Soderberg, 1996) is consistent with our present data. In contrast, IC_{50} values reported for human cancer cell lines, HepG2 and HeLa of 0.002%-0.27% TTO respectively (Hayes, 1997) are highly variable, depending on cell type examined. The observation that concentrations of 0.02% and 0.03% TTO significantly reduces viability of M14 human melanoma cells after 48h (Calcabrini, 2004) supports our present data.

Terpinen-4-ol, the most abundant component in TTO, also had a dose- and time-dependent effect against the two murine tumour cell lines, AE17 and B16. Again, this effect was significantly pronounced in AE17 cells with equivalent concentrations of terpinen-4-ol reducing viability 2.5-4 fold more (24-72h) than in B16 melanoma cells. Terpinen-4-ol was only cytotoxic against normal human fibroblasts (HF32), at doses 5-11 fold greater (24-72h) than with AE17 cells and 2-2.8 fold (24-72h) greater compared with B16 cells. Again, the observation that terpinen-4-ol is significantly more cytotoxic to tumour cells than normal cells is very encouraging. Previously reported IC_{50} values for terpinen-4-ol against HepG2 and HeLa cell lines of 0.006%-0.014% (Hayes, 1997) are consistent with our data against AE17 cells but as observed with TTO, are highly variable, depending on cell type examined.

“Mock” TTO was included in this study to confirm the activity of the active components of TTO. It is composed, of: terpinen-4-ol, α - and γ - terpinene, 1,8-cineole and p-cymene at equivalent concentrations found in TTO. The activity of α - and γ - terpinene, 1,8-cineole and p-cymene alone against all cell lines tested was found to be negligible. As TTO consists of approximately 40% terpinen-4-ol and if terpinen-4-ol is indeed the active component in TTO, we would expect approximately twice the concentration of TTO to elicit at least similar cytotoxicity to terpinen-4-ol alone. Similarly if “Mock” TTO and TTO have similar activity it could be suggested that terpinen-4-ol combined with the other 4 other major components, p-cymene, 1,8-cineole, and α - and γ -terpinene are important in TTO’s activity. But if doses of “Mock” TTO and terpinen-4-ol (at approximately half the concentration) have similar activity it could be suggested that terpinen-4-ol is responsible for the activity observed in these cell lines.

In AE17 cells, TTO has similar activity to terpinen-4-ol, and “Mock” TTO. These observations suggest terpinen-4-ol may be the active constituent in TTO’s anticancer activity against AE17 cells. The observation that TTO is more effective in B16 cells compared with “Mock” TTO, and terpinen-4-ol suggests TTO is more efficacious than the single active terpinen-4-ol, even when mixed with four other major components. This indicates that although terpinen-4-ol’s efficacy alone is significant, its mixture with approximately 100 components found in TTO is more significant in TTO’s anticancer activity against B16 cells.

If the efficacy of terpinen-4-ol is a predictor of TTO’s efficacy against individual cell lines, this could explain the significant difference observed between TTO’s efficacy in tumour cell lines tested versus the normal cell line. The overall significant difference in response following TTO, terpinen-4-ol and “Mock” TTO observed between tumour and non-tumour cell lines *in vitro* suggests TTO kills rapidly

dividing cells more readily than slower growing non-cancerous cells, a feature of many chemotherapeutic agents in use today.

Apoptosis and necrosis induced by TTO and terpinen-4-ol

Combining fluorescent microscopy using Hoechst, annexin-V-FITC/PI staining with analysis by FACS of double staining of cells with annexin-V-PE/PI, allowed the identification and quantification of the mode of cell death induced in AE17 and B16 cells following treatment with TTO and terpinen-4-ol.

AE17 cells treated with TTO for 24 and 48h (0.04%) induced significant necrosis exhibited by cell swelling, nuclear swelling and cell lysis and significant but lower levels of apoptosis. Only with an increased exposure time of 48h and increased concentration to 0.06% was significant necrosis evident in B16 cells, with negligible apoptosis. This correlates well with MTT assay data reported here that indicates AE17 cells are more susceptible to TTO than B16 cells. There is only one publication demonstrating apoptosis induced by TTO in a cancer cell line. Specifically, 0.02% TTO induced ~50% apoptosis in human M14 melanoma cells following a 48h exposure time (Calcabrini, 2004). However, the observation that TTO induced AE17 cell necrosis with increased concentrations and increased exposure times is consistent with multiple studies confirming TTO's mechanism of action involves loss of membrane integrity reviewed in (Carson, 2006).

Treatment with 0.04% terpinen-4-ol displayed similar levels of necrosis in AE17 cells after 24 and 48h exposure and again lower levels of apoptosis with insignificant levels of necrosis and apoptosis in B16 cells. "Mock" TTO (0.04%) displayed extremely similar levels of necrosis and apoptosis in AE17 cells after 24h, but with increased exposure time to 48h, levels of necrosis and apoptosis did not increase; this was also the case in B16 cells where "Mock" TTO induced similarly low levels of apoptosis and necrosis compared with TTO after both 24h and 48h. Only a single study has demonstrated significant apoptosis with terpinen-4-ol. Treatment with 0.01% terpinen-4-ol elicited over 30% apoptosis in an Adriamycin-resistant M14 human melanoma cell line after 48h (Calcabrini, 2004), however this effect was not observed in normal M14 human melanoma cells with only ~2% apoptosis induction following the same concentration and exposure time with terpinen-4-ol (Calcabrini, 2004).

It is not unusual to observe highly variable levels of sensitivity or varying modes of action following treatment of cell lines. Alpha-hederin, a triterpene saponin found in numerous plants, at a concentration of 10.7 μ M after 24h induced up to 73% apoptosis in P388 murine leukaemia cells (Swamy and Huat, 2003), whereas at similar concentrations was found to induce low level apoptosis ranging from 8-13% apoptosis with 11-47% necrosis in human cancer cell lines including A549 and HEp-2 cells (Rooney and Ryan, 2005).

Interestingly, with the addition of staurosporine, a protein kinase inhibitor, which induces apoptosis in numerous cell types, little apoptosis was observed in either AE17 or B16 cells; this could explain the low levels of apoptosis observed following TTO and terpinen-4-ol. This also agrees with the observation that serum starvation failed to induce significant apoptosis in either AE17 or B16 cells (data not shown). This could be indicative that these cell lines might display apoptosis resistance.

Consistently, B16 cells demonstrate a higher resistance to terpinen-4-ol than AE17 cells as observed in cytotoxicity measurements here using the MTT assay. Due to the low levels of cell death induced, that were inconsistent with cytotoxicity indicated by the MTT assay; other potential mechanisms of action were investigated.

Cell cycle arrest induced by TTO and terpinen-4-ol

TTO and terpinen-4-ol induced significant cell cycle arrest in both AE17 and B16 cell lines. Specifically, significantly greater cell cycle arrest was observed in B16 cells when compared with AE17 cells with both TTO and terpinen-4-ol. Interestingly, cell phase arrest appeared to be dose- and time-dependent. With a 12 and 24h exposure of TTO and terpinen-4-ol, a G0/G1 arrest was observed

in B16 cells, resulting in the delay of the transition from G0/G1 to S phase which is apparent as a decrease in cells in S phase. This pattern was also apparent in AE17 cells but to a much lower extent. This arrest in G0/G1 was again observed with increased concentrations of TTO but was also coupled with G2/M phase arrest. At increased exposure times to 48h TTO induced G2/M arrest. In both cell lines cell cycles there was an apparent shift in this effect following treatment with TTO. At higher doses of TTO in both cells lines after 48h, cell cycle arrest in G0/G1 was inhibited and was significantly reduced compared with control cells, with a slight increase in S phase but concomitant with significant arrest in G2/M phase cells. This mechanism of action has yet to be reported in the literature. The observation that potential chemotherapeutic agents induce cell cycle arrest that is dependent on exposure time is consistent with the current literature. The cells analysed for changes in cell cycle distribution were only gated 2N/4N populations; meaning dead or dying cells which would be observed as a sub-G1 peak were not included. Therefore, any observation of cell cycle arrest was not of dying cells and so changes in cell cycle arrest can be attributed as a mechanism of action that is induced besides cell death by apoptosis or necrosis but it is noted that the arrest in cell cycle could lead to apoptosis/and or necrosis. Taken together, the low levels in cell cycle arrest but high levels in necrosis of AE17 cells is consistent with reduced viability observed by MTT assay; conversely, low levels of apoptosis and necrosis observed but coupled with higher levels in cell cycle arrest are consistent with the reduction in viability observed by MTT assay in B16 cells.

Curcumin, the active constituent from turmeric induced cell cycle arrest in G0G1 and G2/M in ECV304 cells (Park, 2002). The observation that TTO and terpinen-4-ol both cause a blockade in G2/M dividing cells but also prevent quiescent cells from entering the cell cycle is very encouraging, as this is a mode of action is demonstrated in many chemotherapeutic agents including paclitaxel, an antitumour agent used extensively worldwide.

***In vivo* experiments**

TTO induces tumour growth inhibition and induces tumour regression

The observation that TTO in a 3% and 10% solution in DMSO applied topically to subcutaneous tumours of malignant mesothelioma can induce tumour growth inhibition and even induce tumour regression is extremely encouraging. DMSO is known to enhance penetration of substances through the skin. It appears that this formulation is critical to induce tumour regression. Treatment was limited to 4 days of 10% TTO and 16 days of 3% TTO due to skin irritation. Tumours did relapse upon cessation of treatment in the majority of mice in the treatment groups. Whilst reducing TTO concentration from 10% to 3% in DMSO did allow increased treatment time from 4 to 16 days as soon as treatment was ceased tumours began to regrow.

In order to overcome this apparent dryness which was limiting the treatment regime; a 3% Novasel® gel was assessed. This is a thermo-reversible poloxamer gel which contains vitamin E but importantly prevents the evaporation of TTO from the skin; hence allowing the TTO to keep in contact to the area for greater time periods appears. Treatment ensued for 16 days with the gel which inhibited tumour growth during treatment and continued to inhibit tumour growth for a further 6 days post treatment cessation. However, no tumour regression was observed.

There have been no studies to elucidate whether any TTO can actually penetrate the skin in this gel form. It is interesting to note that perhaps DMSO allows significant amounts of TTO's components to penetrate the skin and if this is not the case with the Novasel® gel, although the eradication of skin irritation may be cosmetically favourable, the failure of the gel to allow the TTO to penetrate could explain why there is only tumour growth inhibition and no tumour regression upon treatment with the gel. It should also be noted that the skin irritation that ensues with TTO in DMSO may be necessary in order for tumour regression to occur, as no skin irritation and no tumour regression was observed following treatment with the 3% Novasel® gel. It could be speculated that the induced skin irritation induced by TTO in DMSO activates an inflammatory response which could perhaps be responsible for the clearance of the mesothelioma tumour.

The inhibition of tumour growth observed following treatment of topically applied 10% and 3% TTO to subcutaneous tumours of malignant melanoma are also extremely encouraging. The retardation in B16 tumour growth following 10% TTO in DMSO but the limitation to 4 days treatment due to skin irritation led us to again examine lower doses of 3% TTO in the 2 formulations; DMSO and Novasel® Gel.

It should be noted that these murine malignant melanoma (B16) tumours grow at an extremely aggressive rate compared to AE17 mesothelioma tumour growth. B16 control tumours reach experimental endpoints of $\sim 100\text{mm}^2$ at ~ 8 days post tumour inoculation compared AE17 control tumours which reach $\sim 100\text{mm}^2$ at ~ 28 post tumour inoculation. It is for this reason that non-established but inoculated B16 tumours were also assessed with the treatment regime so as to have more time to observe TTO's possible antitumour efficacy.

The 3% TTO in DMSO and 3% TTO Novasel® gel had very similar action against the B16 tumours; both induced tumour growth inhibition but without tumour regression.

The tumour retardation observed with low concentrations of the 3% TTO gel in non-established inoculated B16 tumours, led to the elucidation of whether increased doses of TTO to 5% but in a Gel and Cream formulation would enhance the B16 retardation or perhaps induce tumour regression. These two formulations (5% Gel and 5% cream) were administered twice daily on established (9mm^2) B16 melanomas in order to approximate the concentration of TTO found in the successful tumour retardation experiments using 10% TTO in DMSO. No tumour growth inhibition was observed between the tumours treated with the control gel or the 5% TTO gel or cream formulations; no toxicity to the skin was observed; confirming that formulation was vital for the successful induction of tumour growth inhibition.

In an attempt examine whether increased doses of TTO up to 15% in the Novasel® gel could induce tumour regression or retardation of B16 tumours, we treated non-established but inoculated tumours with TTO in the Novasel® gel at varying concentrations of 5%, 10% and 15%. Daily treatments with a lower concentration of TTO (3%) in a gel formulation have been previously shown to inhibit tumour growth of both AE17 and B16 tumours.

None of the TTO gel preparations resulted in any tumour growth inhibition in the B16 melanoma model. No skin irritation was observed as a result of the treatments. It should be noted that Novasel had to change the formulation of the previously successful 3% TTO gel in order to dissolve higher concentrations of TTO. Unfortunately, all 5-15% gels were unable to absorb adequately into the skin. It is unclear how much of the thicker, thermo-reversible, higher concentration gels, if any, penetrated the skin of the mice in this experiment and perhaps this is why no concentration of the gel elicited an antitumour effect.

Other studies of potential antitumour topical agents from plants include a topical experimental chemotherapeutic agent, 3-ingenyl-Angelate from *Euphorbia peplus* for treatment of murine subcutaneous tumours of B16 melanoma, UV induced squamous carcinoma and Lewis lung carcinoma. $18\mu\text{g}$ of the component induced tumour regression with just 3 topical treatments (Ogbourne, 2004) but with skin irritation similar to that found upon our TTO treatment regime. Its antitumour mode of action involves necrotic cell death (Ogbourne, 2004) and the migration of neutrophils (Challacombe, 2006) associated with an inflammatory response. This is very encouraging when examined alongside the data presented here, as necrotic cell death has been observed as the primary mode of action of TTO against AE17 cells, and an important mode of action coupled with cell cycle arrest in B16 cells.

The observation that TTO in our treatment regime of DMSO induces skin irritation and as speculated above, likely induces an inflammatory response could be important in inducing its tumour inhibition and regression. These data are extremely encouraging; the observation that a topical treatment of just 4 applications can significantly reduce an aggressive mesothelioma solid tumour and retard the growth of a highly aggressive melanoma solid tumour warrant extensive future investigations of the effect of TTO on *in vivo* cancer models.

6. Implications and Recommendations

This study has elucidated some *in vitro* mechanisms of action of TTO and terpinen-4-ol against tumour cells and has demonstrated *in vivo* antitumour activity of TTO against murine subcutaneous tumours. These data have not previously been reported.

TTO and terpinen-4-ol are significantly cytotoxic in a dose- and time- dependent manner to tumour cells *in vitro* and are ~2-fold more efficacious towards AE17 mesothelioma tumour cells than to B16 melanoma tumour. Moreover, TTO and terpinen-4-ol have significantly less cytotoxic efficacy towards normal human fibroblasts. This is highly encouraging. The apparent lack of efficacy of terpinen-4-ol observed in HF32 cells could suggest explain why there is such a differential effect between the tumour cell lines tested versus the normal cell line. We suggest that terpinen-4-ol's cytotoxic activity is the important component driving TTO's cytotoxicity because this component is less active against normal cells versus tumour cells.

TTO and terpinen-4-ol appear to elicit their mechanism of action *in vitro* against AE17 cells primarily through necrotic cell death with low level apoptosis, in a dose- and time-dependent manner, with some levels of cell cycle arrest in G0/G1 but at higher exposure times arrest in G2/M. TTO and terpinen-4-ol appear to elicit their mechanisms of action against B16 cells primarily through significant cell cycle arrest in G0/G1 and at increased exposure times arrest in G2/M, with lower levels of necrosis.

The observation that TTO in a 3% and 10% solution in DMSO and 3% TTO in Novasel® gel applied topically to subcutaneous tumours of malignant mesothelioma and melanoma can induce tumour growth inhibition; and even induce tumour regression (3% and 10% TTO in DMSO) in AE17 tumours is extremely encouraging. The formulation of the TTO appears to be vital to the success of the treatment. TTO in DMSO preparations, though the most effective at inhibiting tumour growth, are also the most toxic to the animal resulting in significant skin irritation. This skin irritation does however completely resolve following cessation of treatment. TTO in gel does not result in any skin irritation but fails to induce tumour regression. Never has any study reported anti-tumour *in vivo* efficacy of TTO in any model.

The fact that TTO is significantly more cytotoxic as assessed by our *in vitro* studies against AE17 tumour cells than B16 tumour cells, and displays a differential mechanism of cytotoxic action; taken together with our *in vivo* observations that TTO induces not only tumour growth inhibition as seen in both tumour models but also significant tumour regression of only AE17 tumours, corroborates the importance of a combination of *in vitro* coupled with *in vivo* investigations. It could be speculated that the high levels of necrosis demonstrated *in vitro* in AE17 cells and tumour regression seen *in vivo* in AE17 tumours are in some way linked and perhaps is one of the mechanisms that TTO induces tumour regression *in vivo*.

Further studies must work towards a formulation of TTO which is both growth inhibitory and induces tumour regression that is well tolerated. Tumours also resume tumour growth post cessation of treatment. Future TTO preparations may also need to be prepared such that they can be administered over a longer period of time in order to allow more prolonged tumour growth inhibition and tumour regression. Alternately, a preparation that can induce a complete and long-lived tumour growth regression would be ideal. The *in vivo* mechanisms of action need to be thoroughly investigated specifically examining possible direct and indirect effects of TTO.

7. References

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Anticancer Activity of Tea Tree Oil

by Dr Sara J Greay, Dr Christine Carson, Dr Manfred Beilharz,
Mr Haydn Kissick, Dr Demelza Ireland and Professor Thomas V Riley

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The aims of this study were to examine the in vitro anticancer efficacy of Tea Tree Oil and its components against cancer and normal cell lines and to examine the in vivo efficacy of TTO as a potential antitumor agent using mouse mesothelioma and mouse melanoma models.

This report discusses data obtained on the in vitro activity of Tea Tree Oil, specifically by significantly reducing viability of malignant mesothelioma and melanoma cells compared with normal human fibroblasts cells in a dose- and time-dependent manner.

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