Topically applied *Melaleuca alternifolia* (tea tree) oil causes direct anti-cancer cytotoxicity in subcutaneous tumour bearing mice

Demelza J. Ireland¹,*, Sara J. Greay¹, Cornelia M. Hooper¹, Haydn T. Kissickᵇ, Pierre Filionᶜ, Thomas V. Rileyᵃ,ᵇ, Manfred W. Beilharzᵃ

¹School of Pathology and Laboratory Medicine (MS04), Faculty of Medicine, Dentistry and Health Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia
ᵇDepartment of Surgery, Beth Israel Deaconess Medical Centre, Harvard Medical School, Boston, MA, USA
ᶜMicrobiology and Infectious Diseases, PathWest Laboratory Medicine WA, Queen Elizabeth II Medical Centre, Nedlands, WA 6009, Australia

**Abstract**

**Background:** *Melaleuca alternifolia* (tea tree) oil (TTO) applied topically in a dilute (10%) dimethyl sulphoxide (DMSO) formulation exerts a rapid anti-cancer effect after a short treatment protocol. Tumour clearance is associated with skin irritation mediated by neutrophils which quickly and completely resolves upon treatment cessation.

**Objective:** To examine the mechanism of action underlying the anti-cancer activity of TTO.

**Methods:** Immune cell changes in subcutaneous tumour bearing mice in response to topically applied TTO treatments were assessed by flow cytometry and immunohistochemistry. Direct cytotoxicity of TTO on tumour cells in vivo was assessed by transmission electron microscopy.

**Results:** Neutrophils accumulate in the skin following topical 10% TTO/DMSO treatment but are not required for tumour clearance as neutrophil depletion did not abrogate the anti-cancer effect. Topically applied 10% TTO/DMSO, but not neat TTO, induces an accumulation and activation of dendritic cells and an accumulation of T cells. Although topical application of 10% TTO/DMSO appears to activate an immune response, anti-tumour efficacy is mediated by a direct effect on tumour cells in vivo. The direct cytotoxicity of TTO in vivo appears to be associated with TTO penetration.

**Conclusion:** Future studies should focus on enhancing the direct cytotoxicity of TTO by increasing penetration through skin to achieve a higher in situ terpene concentration. This coupled with boosting a more specific anti-tumour immune response will likely result in long term clearance of tumours.

© 2012 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.

1. **Introduction**

Topical chemotherapy represents a safe and relatively inexpensive treatment approach for skin cancers. Preclinical and clinical studies of plant derived terpene agents, including ingenol mebutate derived from the sap of *Euphorbia peplus* [1] and *Melaleuca alternifolia* (tea tree) oil [2], have demonstrated the potential for these topical chemotherapeutics to exert a rapid anti-cancer effect after a relatively short treatment protocol. Long treatment regimes, incomplete clearance rates, poor cosmetic outcomes and systemic toxicity have unfortunately limited patient satisfaction with currently approved topical chemotherapeutics such as imiquimod and 5-fluorouracil [3,4].

Tea tree oil (TTO), abundant in terpenes, has been well characterised as an antimicrobial agent [5]. Its use as a topical treatment has been reported in numerous clinical trials and has been rigorously examined for toxicity and safety. TTO formulations containing 5–100% oil have been patch tested for up to 21 days in large cohorts of patients with no or few irritancy reactions recorded. Those irritant reactions that do occur are significantly reduced with more dilute preparations. Moreover, allergic reactions to topical TTO are negligible with fresh, and appropriately stored TTO (reviewed in [6]). More recently, TTO and some of its components have been examined for anti-tumour activity [7–10]. When applied topically in a specific formulation to fully immune-competent, subcutaneous, tumour-bearing mice, TTO showed *in vivo* anti-tumour activity [2]. Four, daily, topical treatments of 10% TTO/DMSO caused subcutaneous AE17 mesotheliomas in mice to regress for a period of 10 days and significantly retarded the growth of subcutaneous B16-F10 melanomas. We previously reported this anti-tumour effect of topical 10% TTO/DMSO to be accompanied by skin irritation similar...
to other topical chemotherapeutic agents but, unlike other approved
topical chemotherapeutics, 10% TTO/DMSO induced skin irritation
resolved quickly and completely. Furthermore, by histological
analysis we showed that topical 10% TTO/DMSO caused an influx
of neutrophils into the epidermis and dermis with no evidence of
systemic toxicity.

We originally proposed that local immune activation in
response to topical treatment with 10% TTO/DMSO may be by a
process similar to that of ingenol mebutate which induces a
neutrophil mediated, antibody dependent, cellular cytotoxic
eradication of residual tumour cells [11]. In this report, we
document evidence suggesting that although topical application of
10% TTO/DMSO activates a local immune response, our evidence
shows that the anti-tumour mechanism of action underlying the
anti-tumour effect is a result of direct cytotoxicity.

2. Materials and methods

2.1. Cells and cell culture

AE17 murine mesothelioma cells were derived from asbestos
induced primary tumours of syngeneic C57BL/6J mice [12]. All cells
were maintained at 37 °C in 5% CO₂ in 75 cm² or 225 cm² tissue
culture flasks in supplemented RPMI-1640 media and subcultured
as previously described [2,13].

2.2. Mice

Female C57BL/6J mice between 6 and 8 weeks of age were
obtained from the Animal Resources Centre (Perth, Australia) and
maintained under SPF housing conditions (Animal Care Unit,
University of Western Australia) in tinted Perspex cages with filter
lids on a 12-h day/night cycle. All animal work was carried out in
accordance with the guidelines of The National Health and Medical
Research Council of Australia and with the approval of the
University of Western Australia’s Animal Ethics Committee (AEC
RA/100/728).

2.3. Tumour cell implantation and growth monitoring

1 × 10⁷ AE17 cells/100 μl PBS/mouse were implanted using a
100 μl glass syringe (26½ gauge needle) sub-cutaneously (s.c.)
onto the shaved, right hand flank of syngeneic C57BL/6J mice as
previously described [13]. Tumour development was monitored
visually and physically by palpating the tumour implantation site.
Tumours were measured using a microcalliper to obtain two
tumour diameters taken at right angles to each other; these were
multiplied to obtain a tumour area (mm²).

Simultaneously, animals were monitored daily for any loss of
overall condition by checking for: hunching, ruffled coat, loss of
body weight, bloating, slowed movement and seclusion. Humane
euthanasia by methoxyflurane inhalation followed by cervical
dislocation was carried out when tumour area reached 100 mm² or
at predetermined experimental endpoints.

2.4. TTO and topical treatments

TTO compliant with the International Standard ISO4730 was
kindly provided by P. Guinane Pty. Ltd., Chinderah, NSW (Batch
A352) [2]. A 10% solution of TTO was made by dissolving TTO in
DMSO Hybri-max (Sigma Chemical Co., St Louis, MO). All TTO
solutions were kept in airtight containers in the dark, with regular
stability testing to ensure normal levels of terpinen-4-ol and 1,8-
cineole.

Mice with shaved flanks and established tumours measuring
9 mm² (approximately 12–14 days post tumour inoculation) were
treated topically with either 10% TTO/DMSO (50 μl), neat TTO
(5 μl: an amount equivalent to that found in 10% TTO/DMSO), or
vehicle control (50 μl: 10% H₂O/DMSO) applied by pipetting the
required volume onto the tumour and surrounding skin and gentle
rubbing [2]. Treatments were applied once daily (at the same time
each day) for 4 consecutive days. Mice were monitored daily for
skin irritation resulting from topical treatments and under our AEC
regulations were humanely euthanised if a severe skin irritation
score of >3 (average: dryness score + erythema score + eschar
formation score) was observed.

2.5. Antibody depletions

A total of 100 μg of Gr-1 mAb (RB6.8C5, Monoclonal Antibody
Facility, Western Australian Institute for Medical Research) was
administered i.p. to mice on days −1, +1, +3 and +5 to deplete
neutrophils (topical treatments were applied concurrently on days
0–3). Control mice were administered with an isotype-matched
control antibody. Samples of blood (1 μl) collected via a tail vein
nick 1 day post Gr-1 mAb depletion were smeared onto glass
microscope slides and stained with haematoxylin and eosin (H&E),
as well as stained for Nimp-1 for flow cytometry, and the numbers of
neutrophils were counted. As determined, >90% systemic
neutrophil depletion was achieved.

2.6. Flow cytometry

Preparations of single cell suspensions were made from tumours
(including overlying skin) and tumour draining lymph nodes
(TDLNs). Tumour/skin and TDLNs were surgically removed and
minced finely at room temperature in 500 μl of un-supplemented
RPMI. Following the Indianapolis, IN addition of 1.4 U (12.5 μl)
liberase blendzyme 3 (Roche, Indianapolis, IN) and 1000 kU (13.5 μl)
DNase 1 (Sigma Chemical Co., St Louis, MO), samples were
digested by mixing on a rotating wheel at room temperature
for 20 min before centrifugation at 1500 × g for 3 min. Resulting cell
homogenates were pushed through fine wire mesh to break down
any remaining clumps. The single cell suspension was washed twice
with 500 μl wash buffer (PBS containing 5% fetal calf serum (FCS)
and 5 mM Na₂HPO₄) and centrifuged at 1500 × g for 3 min before
staining with fluorochrome-conjugated antibodies for flow cyto-
metric analysis. Flow cytometric staining for cell surface markers
MHCI, Ly-6G (Gr-1), CD4, CD8, CD11c, CD69, CD80, and CD86
(all purchased from eBioScience, San Diego, CA), as well as Nimp-1
(Abcam, Cambridge, MA) was analysed using a BD FACSCanto™
benchtop flow cytometer (BD Biosciences, San Jose, CA), recording
at least 100,000 events per sample. Data were then analysed using
FlowJo software (Treestar Inc., San Carlos, CA).

2.7. Histology

For histology and immunohistochemistry, mice were euthanised
following 1 or 3 topical treatments with either 10% TTO/DMSO or
vehicle and embedded in O.C.T. compound (optical cutting
temperature, Thermo-Shandon, Pittsburgh, PA). Frozen cross-sections
of tumour plus overlying skin were prepared at 10 μm thickness. For
lipid staining, frozen cross sections were rehydrated in PBS and
stained for 20 min in a 0.5% Oil red-O (Merck, Darmstadt, Germany)
solution in Dextrin (BDH, US). After rinsing, the sections were
counterstained with haematoxylin for 20–30 s and mounted with the
sections analysed using a Digital Slide Scanner and ImageScope
software version 10.2.2.2352 (Aperio Technologies Inc., CA). For
visualisation and localisation of neutrophils (Nimp-1), the frozen
sections were rehydrated and blocked with 1 × PBS supplemented
with 1% BSA (bovine serum albumin) (Sigma Chemical Co., St Louis,
MO), 0.5% Triton X-100 (Sigma Chemical Co., St Louis, MO), 2 mM
EDTA (Sigma Chemical Co., St Louis, MO) and 10% goat serum (Invitrogen, Frederick, MD). The blocked sections were incubated with Nimp-1 (Abcam, Cambridge, MAA) and conjugated with secondary goat anti-rabbit AlexaFluor488 antibody (Invitrogen, Eugene, OR). Tissue sections were washed in 1x PBS supplemented with 1% BSA and 2 mM EDTA and counterstained with 1 μg/ml Hoechst 33342 (Sigma Chemical Co., St Louis, MO) for 1 min. The stained preparations were cover slipped using ImmuMount (Thermo Shandon Ltd, UK) and images were captured using an inverted AI confocal microscope and NIS-elements imaging software (Nikon, Japan).

2.8. Transmission electron microscopy

For transmission electron microscopy (TEM), tumours were excised and manually sectioned, avoiding the necrotic tumour core. Tumour sections were fixed overnight in 1 ml of 2.5% glutaraldehyde in cac buffer (0.05 M, pH 7.0) and prepared for TEM as previously described [13]. Tumour samples were examined on a CM10 transmission electron microscope (Phillips, Netherlands) fitted with MegaViewIII Soft Imaging System (Olympus, Japan). All figures were prepared in Photoshop Elements 9 (Adobe Systems Incorporated, San Jose, CA).

2.9. Statistics

All data fulfilling criteria for normal distribution are presented as mean ± SD, or mean ± SE for pooled independent experiments. Significant differences as indicated were calculated using the Student’s t tests (p < 0.05), and two-way ANOVA test (p < 0.01 or p < 0.001). Skewed data, not following normal distribution, is presented using the median and range. For significance, the two-tailed Mann Whitney test (p < 0.05) and the ANOVA equivalent KrusWallis test were used. All statistical tests and figures were prepared using the Graph Pad Prism software version 5 (GraphPad Software, San Diego, CA).

3. Results

3.1. Neutrophil accumulation in the skin following topical 10% TTO/DMSO treatment

Flow cytometry of single cell suspensions of the skin/tumour of mice bearing established s.c. AE17 tumours treated topically with 50 μL of 10% TTO/DMSO for 4 consecutive days demonstrated a significant 200-fold (p < 0.001) increase in the number of neutrophils by comparison of medians with vehicle control treated tumours. A 230-fold (p < 0.01) increase in neutrophils was observed following 10% TTO/DMSO compared to neat TTO treated tumours or untreated tumours at days 3 and 6 post treatment initiation (Fig. 1A). The peak accumulation of neutrophils at day 3, confirmed in 2 further independent experiments (Fig. 1B), was followed thereafter by a decrease in most animals as indicated by the increase in variance in neutrophil numbers on day 6. This increase in neutrophil number correlated well with the peak in skin irritation and the previously reported regression of AE17 s.c. tumours [2]. By day 10 post treatment, neutrophil numbers returned to normal, pre-treatment levels. This also coincided with the previously observed resumption of AE17 s.c. tumour growth/tumour relapse following cessation of topical 10% TTO/DMSO treatment [2].

In situ analysis of the populations of neutrophils in topical 10% TTO/DMSO treated skin and s.c. AE17 tumours was performed by confocal fluorescence microscopy of cross-sections of tumour/skin harvested from mice 3 days post treatment initiation. Visualisation revealed the presence of high numbers of neutrophils (Nimp-1+) in the skin and the upper dermal layer but not infiltrating the topical 10% TTO/DMSO treated s.c. tumour (Fig. 1C). Very few neutrophils were evident in the vehicle control treated tumour or skin overlying the tumour.

3.2. Topical treatment with 10% TTO/DMSO increases dendritic cell (DC) and T cell numbers in TDLNs

Based on the dynamics of the acute neutrophil response, the DC and T cell response to topically applied 10% TTO/DMSO was also investigated in TDLNs of mice bearing established s.c. AE17 tumours. TDLNs were harvested and analysed for changes in both DC number and activation (defined by an increase in expression of the CD80 and CD86 co-stimulatory molecules). Throughout the treatment period, and up to 6 days post treatment initiation with topical 10% TTO/DMSO, the number of DCs in the TDLN of s.c. tumour bearing mice was significantly higher than that found in neat TTO, vehicle-control and untreated tumour bearing mice (Fig. 2A and C). A similar increase in the number of DCs 3 days post TTO treatment initiation was also observed in non-tumour bearing naive mice (data not shown). In addition, topical 10% TTO/DMSO significantly increased the CD80 expression by DCs in TDLNs during and up to 6 days post treatment initiation, with CD80 expression returning to normal levels by day 10 (Fig. 2B and D). A similar trend was also observed for the DC activation marker CD86 (data not shown).

T cell activation by DCs is an immune event required for T cell mediated tumour clearance and the creation of immunological memory. We therefore examined the number of CD4+ and CD8+ T cells in TDLNs 3 days post topical 10% TTO/DMSO treatment initiation. Whilst topical treatment with 10% TTO/DMSO increased the numbers of both CD4+ and CD8+ T cells in TDLNs of AE17 s.c. tumour bearing mice (Fig. 2E and F), neither the CD4+ nor the CD8+ T cell populations appeared to show increased expression of the activation marker CD69 (data not shown). A T cell mediated clearance of tumours was also deemed unlikely as a double depletion of CD4+ and CD8+ T cells did not abrogate the anti-cancer effect of topically applied 10% TTO/DMSO (data not shown).

3.3. Neutrophils appear to be involved in skin irritation but not tumour regression after topical 10% TTO/DMSO treatment

The functional role of neutrophils in DC activation and T cell changes were examined next. Immunocompetent mice were depleted of neutrophils using a monoclonal antibody against Gr-1 administered i.p. every second day, starting 1 day prior to the initiation of topical 10% TTO/DMSO treatment. Systemic depletion of neutrophils was assessed over the treatment period in the peripheral blood by both microscopy (Fig. 3F) and flow cytometry (data not shown) as well as in the tumour and overlying skin by both flow cytometry (data not shown) and immunohistochemistry using the neutrophil marker, Nimp-1 (Fig. 3F). Neutrophils were absent in the blood and in the epidermis of neutrophil (Gr-1)-depleted animals confirming a neutrophil depletion of >90% was achieved throughout the monitored time frame.

The flow cytometric analysis of DC populations in TDLNs of neutrophil depleted AE17 s.c. tumour bearing mice treated with topical 10% TTO/DMSO revealed that the increase in the number of DCs (above vehicle treated AE17 s.c. tumour bearing mice) and the increase in CD80 expression by DCs in TDLNs occurred equally and independently of neutrophils (Fig. 3A and B). In contrast, the previously observed CD4+ and CD8+ T cell accumulation in TDLNs of immune competent AE17 s.c. tumour bearing mice in response to topical 10% TTO/DMSO treatment was less obvious when neutrophils were depleted (Fig. 3C and D). Finally, as shown in Fig. 3E, the depletion of neutrophils did not abrogate the anti-tumour efficacy of topically applied 10% TTO/DMSO, as tumours regressed independent of the presence of neutrophils. Analysis
**Fig. 1.** Topical 10% TTO/DMSO treatment induces an accumulation of neutrophils in the skin overlying s.c. AE17 tumours. (A) The number of neutrophils in the s.c. AE17 tumour and skin above the tumour (pooled for analysis) following daily, topical treatment for 4 days with 10% TTO/DMSO (50 µl), neat TTO (5 µl), vehicle control 10% H2O/DMSO (50 µl), or no treatment (●), on days 1, 3, 6 and 10 post treatment initiation in C57BL/6J mice bearing established (9 mm²) s.c. AE17 tumours. The data are shown as median ± range of the number of neutrophils per gram skin/tumour analysed by flow cytometry (n = 5 mice). * Indicates statistically significant (***p < 0.001, ****p < 0.0001) from vehicle control by two-way ANOVA (KrusWallis) test. (B) For increased statistical confidence, the number of neutrophils was compared between the s.c. AE17 tumour and the skin overlying the tumour following daily, topical treatment for 3 days with 10% TTO/DMSO or the vehicle control (10% H2O/DMSO) using 12 mice from 3 independent experiments. The data are shown as median and *** indicates a p-value < 0.0001 by two-tailed Mann–Whitney test. (C) C57BL/6J mice bearing established s.c. AE17 tumours were treated topically with 10% H2O/DMSO (left) and 10% TTO/DMSO (right) for three consecutive days with cross-sections of tumour/skin stained for neutrophils using Nimp-1 (green). Tissue sections were counter stained with Hoechst 33342 (blue).

**Fig. 2.** Topical 10% TTO/DMSO causes DC and T cell accumulation in tumour draining lymph nodes of AE17 s.c. tumour bearing mice. C57BL/6J mice with established (9 mm²) subcutaneous AE17 tumours treated topically daily for 4 days with 50 µl of 10% TTO/DMSO (●), neat TTO (▲), 50 µl vehicle control 10%H2O/DMSO (■) and no treatment (▲). On days 1, 3, 6 and 10 after the initiation of treatment, the (A) number of CD11c+/MHCIIC+ cells (DCs) per TDLN and (B) fold increase in the CD80 mean fluorescence intensity (MFI) of DCs was assessed by flow cytometry. (C) DC number, (D) DC activation, (E) CD4+ T cell and (F) CD8+ T cell numbers measured in TDLNs 3 days post treatment initiation with daily topical 10% TTO/DMSO or the vehicle control. Data are presented as the median ± range of ≥3 mice per treatment group from 3 (DC) and 2 (T cells) independent experiments (*p < 0.05, **p < 0.01, ***p < 0.0001 by two-tailed Mann–Whitney test).
of skin irritation during this experiment revealed a less pronounced scabbing of the skin in neutrophil depleted animals after 10% TTO/DMSO (Fig. 3G) plus a shorter healing time.

3.4. The anti-tumour efficacy of topical 10% TTO/DMSO is mediated by a direct effect of TTO on tumour cells in vivo

Based on these functional studies there was no evidence to show that TTO induced a T cell specific response against the AE17 tumours nor that neutrophils contributed to more than skin inflammation observed at the treatment site. This led to the investigation of the direct effect of TTO on tumour cells in vivo. Our group has previously demonstrated that TTO exerts direct cytotoxic effects on AE17 and B16-F10 tumour cells causing low level apoptosis and primary necrotic cell death in vitro, at concentrations non-cytotoxic to non-tumour fibroblasts [13]. In order to test whether TTO exerts direct cytotoxicity on tumour cells in vivo, topical 10% TTO/DMSO treated AE17 tumours were cross-sectioned on days 1 and 3 post treatment initiation and analysed by TEM. AE17 tumour cells in topical 10% TTO/DMSO treated mice revealed changes in both the gross tumour tissue organisation as well as the individual cell ultrastructure when compared to the spatially well organised vehicle control treated tumour cells (Fig. 4A). After topical 10% TTO/DMSO treatment s.c. AE17 tumours progressively lost cellular organisation marked by an increase in intercellular spaces and an accumulation of cell debris, lipids (L) and membrane micelles (Fig. 4A). Moreover, this loss of cellular organisation following TTO/DMSO treatment was more prominent in the upper tumour regions, proximal to the skin (Fig. 4B). Similar to our in vitro observations, fibroblasts adjacent to damaged tumour cells appeared healthy following 10% TTO/DMSO treatment (Fig. 4C). This was also true for lymphocytes identified within tumour sections and skeletal muscle fibres adjacent to tumours (data not shown).

AE17 tumour cells displayed signs of damage and death as early as 1 day post topical 10% TTO/DMSO treatment (Fig. 5). This included the compression of nuclei and contraction of chromatin accumulating on nuclear membranes. Mitochondria showed signs of swelling with increasing disruption of mitochondrial cristae and membranes together with a loss of protein organisation and an occasional fusing with lysosomes. Remnant mitochondria appeared pale and ruptured by day 3 post topical 10% TTO/DMSO treatment initiation. A clear loss of the highly interconnected organisation of ER structures was also visible with ER appearing...
dilated just 1 day after treatment initiation. The ER had almost completely dissolved by day 3 with ribosomes mostly detached. Similarly, cell membranes became less defined with increasing topical 10% TTO/DMSO treatment duration. AE17 cell bodies appeared distorted and ruptured with cellular constituents leaking into the intercellular space. The cytoplasm and mitochondria of the s.c. AE17 tumour cells treated with topical 10% TTO/DMSO became increasingly more electron lucent with repeated exposure to TTO and appeared to contain little metabolites.

The differential tumour cell damage seen in the upper versus the lower regions of tumours (most distant from the treatment site) suggested that the ability of TTO in this formulation to penetrate deep into tumours may be the determining factor in the success of this topical chemotherapy. In order to investigate the localisation of TTO within the skin and s.c. AE17 tumours after topical application of the 10% TTO/DMSO formulation, tumour/skin cross-sections of topical 10% TTO/DMSO treated (Fig. 6A right and B top) and vehicle treated (Fig. 6A left and B bottom) AE17 s.c. tumours
were stained for visualisation of lipophile compounds using Oil red-O stain and light microscopy. Lower magnification (Fig. 6A) revealed strong staining of adipose tissue (AdTi) and the thickened stratum corneum (SC) of AE17 s.c. tumours treated with topical 10% TTO/DMSO. An investigation of the localisation of oils (red) at higher magnification (Fig. 6B) revealed that the viable epidermis (Ep) of mice bearing s.c. AE17 tumours treated topically with 10% TTO/DMSO contained more lipid droplets (LiD) with some droplets penetrating into the sub-epithelial layers as compared to the vehicle control treated tumours. The presence of LiD below the stratum basale of mice treated topically with 10% TTO/DMSO suggested that TTO diffused into the dermal cell layers and s.c. AE17 tumour tissue but that the concentration of TTO within the actual tumour still appeared very low and was undetectable by this method.

4. Discussion

Current topical chemotherapy options for actinic keratoses (precancerous skin lesions/sun spots), and superficial non-melanoma skin cancers, include imiquimod and 5-fluorouracil. Their respective modes of action are (i) TLR mediated immune killing of tumours, in addition to apoptosis in some tumour cell types [14] and (ii) interference with RNA and DNA synthesis leading to cell death in abnormal cells [15,16]. The recently US Food and Drug Authority (FDA) approved ingenol mebutate may offer an alternative as current chemotherapies are often plagued by limitations including long treatment regimes and debilitating skin reactions. After a shorter treatment regime, ingenol mebutate induces primary necrotic tumour cell death together with neutrophil mediated antibody dependent cellular cytotoxicity (ADCC) to prevent tumour relapse [11].

Similar to the history of ingenol mebutate [1], there was anecdotal evidence to suggest that TTO may have anti-cancer efficacy. Over the last 4 years, communications from the general public have included reports that home remedies of topical TTO mixtures are effective at clearing both actinic keratoses and skin tumours; including basal cell (BCC) and squamous cell (SCC) carcinomas. We have thus worked to scientifically validate this anecdotal evidence and have successfully demonstrated both in vitro and in vivo (murine models) that TTO can in fact inhibit tumour cell growth. There are several novel findings in this current preclinical study of the mechanism of action underlying the anti-cancer activity of topically applied TTO in a DMSO formulation. Specifically, it is now clear that topically applied 10% TTO/DMSO causes a direct cytotoxic effect on subcutaneous AE17 tumour cells coupled with an apparent non-tumour specific activation of a local immune response including, at least, neutrophils, DCs and T cells.
Little is known of the effect of topically applied essential oils, especially TTO, with respect to the induction of inflammation or immune infiltration in the skin. Topically applied TTO can suppress both the oedema induced by intradermal injection of histamine [17] and neutrophil accumulation [18]. We noted that skin inflammation is similar in both tumour bearing and non-tumour bearing mice treated with 10% TTO/DMSO and is confined to the upper epidermal layers, specifically the stratum corneum which undergoes thickening and shedding in response to treatment (data not shown). The reduced skin irritation in neutrophil depleted mice suggested the accumulation of neutrophils was related to skin this inflammation and associated with the combination of DMSO with TTO. Skin inflammation and damage to epithelial tissues is known to liberate pro-inflammatory cytokines (e.g., TNF-α and IL-1β) that activate endothelial cells to produce neutrophil attractants (e.g., IL-8) [19,20] with neutrophil granule-derived components, such as azuricin and defensins known to be chemotactic for activated T cells and immature DCs [21] to inflamed tissues [22]. In this study, topical 10% TTO/DMSO treatment was shown to induce the highest increase in neutrophil numbers on day 3 post treatment initiation; the specific timepoint at which the most significant tumour regression is observed together with the peak in DC accumulation and activation in TDLNs and the increase in T cell numbers in TDLNs. Taken together, it was proposed that similar to the anti-cancer effect of ingenol mebutate, neutrophils likely played a role in our AE17 s.c. tumour model to influence the adaptive immune system and thus mediate 10% TTO/DMSO induced tumour regression. However, neutrophil depletion experiments demonstrated that the anti-tumour efficacy of topically applied 10% TTO/DMSO was not dependent on neutrophils; DC numbers still increased and DCs were still activated, suggesting an involvement of alternative immune cells and/or other mechanisms of tumour regression. The increase in neutrophils at the treatment site in response to topical 10% TTO/DMSO treatment, the accumulation and activation of DCs in TDLNs together with an increase in both CD4+ and CD8+ T cell numbers confirmed a local immune cell activation but did not demonstrate an antigen-specific, T cell mediated clearance of tumours; akin to the non-tumour specific mechanism of action of 5-fluorouracil. In fact, a CD4 and CD8 co-depletion study demonstrated that 10% TTO/DMSO cleared established tumours even in a state of reduced T cell function.

Tumour cell death does not however go unnoticed by the innate and adaptive immune responses; in fact, depending on the cell death inducer, some types of tumour cell death have been shown to induce an anti-tumour immune response capable of overcoming tumour-induced immune-suppression [23]. Tumour cell death induced by some chemotherapies and radiotherapies can induce an “eat me” signal associated with the exposure of calreticulin on the cell surface [24]. The secretion of the high-mobility-group box 1 (HMGB1) alarmin protein as a second “danger” signal is likely required for an efficient anti-tumour immune response [25]. It is proposed that in some cases, and potentially in this study in response to topical 10% TTO/DMSO treatment, that there is still some signalling between dying tumour cells and DCs. It is possible that TTO induced direct cell death resulting in secretion of death signals and the activation of DCs thereby explaining the local immune infiltration and activation, including neutrophils [26], seen in response to topical application of 10% TTO/DMSO.

Studies presented here have clearly shown that topically applied 10% TTO/DMSO is directly, and selectively, cytotoxic to AE17 tumour cells in vivo. Our observations suggest that TTO/DMSO causes primary necrosis of AE17 tumour cells in vivo and confirms our previous in vitro study demonstrating that TTO had a dose dependent effect in AE17 cells by inducing low level apoptosis and primary necrotic cell death [13]. TEM demonstrated that in vitro TTO treatment of murine tumour cells targeted primarily mitochondria resulting in mitochondrial membrane disruption, gross swelling and dissolution of internal structures. In vivo we have now shown by TEM a significant impact of topical 10% TTO/DMSO treatment on the integrity of s.c. AE17 tumour cell membranes and membrane-bound cell organelles. The changes in the plasma membrane, cell organelle swelling and disruption of the mitochondrial cristae are suggestive that AE17 cells underwent primary necrosis. Similar changes in cell eukaryotic membranes following TTO treatment have been observed previously in other human and murine tumour cells [13,27] and in AE17 cells in vitro [2]. Scanning electron microscopy of migrating melanoma cells...

**Fig. 6.** TTO localises predominantly to the epidermis following topically applied 10% TTO/DMSO. Representative microscopy images of C57BL/6J mice bearing established s.c. AE17 tumours treated topically, daily for 3 days with 10% TTO/DMSO or vehicle control. (A) Vehicle control (left) and 10% TTO/DMSO (right) treated skin and s.c. AE17 tumour sections on day 3 post treatment initiation. (B) Vehicle control (bottom) and 10% TTO/DMSO treated (top) skin sections at higher magnification. Ep = epidermis, AdTi = adipose tissue, SeGl = sebaceous gland, SkMu = skeletal muscle, LiD = lipid droplet, SC = stratum corneum.
showed them developing chasms in the plasma membrane with surface blebbing and the release of blebs in response to treatment with TTO [7]. TTO and its single terpenes integrate into membranes increasing their fluidity and making them less stable [28–30]. This appears to be concentration dependent and may cause membrane rupture in cancer cells if a critical single or combined terpene concentration is reached; normal fibroblasts and immune cells seem to show a lower tolerance. The upper area of a tumour, proximal to the skin, shows increased tumour cell destruction as compared to the lower parts. This differential effect, and the presence of residual tumour cells, likely explains the 10% tumour relapse seen post cessation of 10% TTO/DMSO treatment [2]. DMSO is also well known to exert similar membrane thinning effects by incorporating into the lipid tails of the bilayer structure of membranes increasing membrane fluidity [31]. This induces transient water pores and, above critical concentrations, a disintegration of cell membranes [32,33]. As topical treatment with DMSO vehicle or neat TTO did not affect tumour growth [2] or immune activation, it is thought that in this case DMSO enhances penetration of TTO through the stratum corneum into the epidermis by altering the cohesive lipid packing [34]. Considering that topical treatment with neat TTO or DMSO vehicle did not demonstrate any visible membrane disruption in this study, it is very likely that the membrane-based cytotoxic effect observed in vivo is due to an interactive or accumulative effect of DMSO and the terpenes of TTO.

It is clear from this research that topically applied dilute TTO in a DMSO formulation induces both local immune activation and a direct cytotoxic effect on tumour cells. Though the exact cascade of events leading to the clearance of s.c. AE17 tumours still remains to be elucidated, it is clear that TTO is directly cytotoxic to these tumour cells. DMSO is used in these studies as a penetration enhancer with penetration shown to correlate with treatment efficacy. Enhancing the direct cytotoxicity of TTO by increasing penetration and in situ terpene concentration is the focus of current research with the formulation of TTO into a more clinically acceptable vehicle also a key priority. The AE17 tumour model used in these studies is particularly aggressive and known to be subject to significant immunosuppression potentially explaining the lack of T cell mediated clearance of tumours [35–37] following TTO treatment. Overcoming this immunosuppression has been the focus of much research in our laboratory and has proved difficult until recently [38]. Topical TTO treatments coupled with such an immune boosting strategy will likely result in long term clearance of tumours. The future demonstration of complete and long term tumour regression will facilitate the translation of topical TTO treatment to the clinic for the treatment of human skin tumours.

Acknowledgements

The authors acknowledge the facilities, scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments. The authors also acknowledge the facilities, scientific and technical assistance of CELLCentral, School of Anatomy and Human Biology, The University of Western Australia, and The M Block Animal Care Unit, The University of Western Australia. The authors would like to thank Dr. Christine Carson for stimulating discussions and UWA Honours students and Sharnice Koek and Vincent Kuek for technical assistance. This work was financially supported by the Rural Industries Research and Development Corporation, ACT, Australia (PRJ-002395) together with Novasel Australia Pty. Ltd., Mudgegeraba, QLD, Australia.

References


