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ORIGINAL ARTICLE

Inhibition of established subcutaneous murine tumour growth with topical *Melaleuca alternifolia* (tea tree) oil

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Abstract
Purpose Systemic toxicity coupled with long treatment regimes of approved topical chemotherapeutic agents such as imiquimod and 5-fluorouracil (5-FU) are limiting. There is now more focus on the potential use of topical terpene agents as skin cancer treatments. Here, we show for the first time that topical *Melaleuca alternifolia* (tea tree) oil (TTO), abundant in terpenes, has in vivo antitumour activity.
Method Topical TTO formulations applied to immunocompetent tumour-bearing mice were assessed for antitumour efficacy by monitoring tumour growth and by histological analysis following treatment.
Results Four, daily, topical treatments of 10% TTO/DMSO regressed subcutaneous AE17 mesotheliomas in mice for a period of 10 days and significantly retarded the growth of subcutaneous B16-F10 melanomas. The antitumour effect of topical 10% TTO/DMSO was accompanied by skin irritation similar to other topical chemotherapeutic agents, but unlike other approved topical agents, quickly and completely resolved. Furthermore, we show that topical 10% TTO/DMSO caused an influx of neutrophils and other immune effector cells in the treated area, with no evidence of systemic toxicity.
Conclusion TTO combined with an effective carrier significantly inhibited the growth of aggressive, subcutaneous, chemo-resistant tumours in immunocompetent mice. Taken together, these findings highlight the potential of topical TTO as an alternative topical antitumour treatment.

Keywords Tea tree oil · Topical chemotherapy · Antitumour · Murine

Introduction

Topical chemotherapy, when combined with surgical excision of skin cancers or as a single therapy, represents a safe, convenient and often inexpensive alternative anticancer treatment. The immune modifier imiquimod and the thymidylate synthase inhibitor 5-fluorouracil (5-FU) have been used successfully as topical treatments for superficial basal cell carcinoma (sBCC) and actinic keratoses. However, systemic toxicity coupled with long treatment regimes of up to 6 weeks are limiting. Recently, studies investigating the antitumor efficacy of topical terpene agents, such as perillyl alcohol (POH) and ingenol-3-angelate, have highlighted the effectiveness and substantially shorter treatment regimes of these plant-derived therapies [1–3]. Ingenol-3-angelate derived from *Euphorbia peplus* induces necrotic cell death and dendritic cell (DC) activation, and increases

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neutrophils at treated sites to prevent relapse of subcutaneous tumours [1, 2, 4]. POH, found in sage, lemongrass and cherries, acts by Ras suppression, apoptosis and cell cycle arrest [5, 6]. POH and ingenol-3-angelate are currently in phase II and phase III clinical trials, respectively.

In vitro studies of *Melaleuca alternifolia* (tea tree) oil (TTO) have demonstrated its effectiveness against human melanoma cells [7]. In addition, we have previously shown that TTO has significant antiproliferative activity against tumour cells through primary necrosis and G1 cell cycle arrest [8]. Numerous studies have shown the effectiveness of topical TTO as an antibacterial and antifungal treatment (reviewed in [9]), yet no study to date has reported on the potential antitumour efficacy of topical TTO. We have successfully used a subcutaneous murine mesothelioma tumour model in immunocompetent mice to investigate the antitumor effect of intratumoural anti-CD25 monoclonal antibody treatment [10, 11]. Moreover, similar subcutaneous tumour models have been successfully used to demonstrate efficacy of other immunotherapies [12, 13].

In this report we show for the first time that, when combined with an effective carrier, once daily for 4 daily topical treatments with TTO has significant activity against aggressive, subcutaneous, chemo-resistant tumours in immunocompetent mice. Furthermore, we show that topical TTO causes an influx of neutrophils in the treated area, with no evidence of systemic toxicity. Taken together, these findings highlight the potential of topical TTO as an alternative antitumour treatment.

Materials and methods

Mice

Female C57BL/6 J 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, The University of Western Australia). 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). Mice treated topically were monitored for dryness, erythema and eschar formation and scored: 0: no irritation, 1: mild irritation, 2: moderate irritation, 3: severe skin irritation. Under our AEC regulations, once mice reached the humane endpoint for tumours (100 mm^2) or an average severe skin irritation score of greater than 3 (average: dryness score + erythema score + eschar formation score), they were euthanised by penthrane inhalation followed by cervical dislocation.

Murine mesothelioma and melanoma cell culture, in vivo implantation and tumour growth monitoring

The AE17 murine mesothelioma and B16-F10 murine melanoma cells used in this study were cultured as previously described [8, 10]. Tumour cells (5×10^5/100 µl PBS B16-F10 cells or 1×10^7/100 µl PBS AE17) prepared for implantation were subcutaneously injected, and tumour growth was monitored as previously described [10].

Tea tree oil

TTO compliant with the International Standard 4730 [14] was kindly provided by P. Guinane Pty. Ltd., Chinderah, NSW. Batch 1216 detailed in [8] and batch A352 which contained the following major components: 37% terpinen-4-ol; 18.6% γ-terpinene; 10% α-terpinene; 3.6% 1,8-cineole and 1.4% ρ-cymene as determined by gas chromatography mass spectrometry carried out by NSW Department of Primary Industries, Diagnostic and Analytical Services, Environmental Laboratory, Wollongbar, NSW, were both used in this study. A 10% stock solution of TTO was made by dissolving TTO in DMSO (Sigma Chemical Co., St Louis, MO, USA). TTO-5C solution was made by mixing the following major components of TTO at concentrations equivalent to their composition in TTO; 40% terpinen-4-ol, 20% γ-terpinene, 10% α-terpinene 5% 1, 8-cineole, 5% ρ-cymene and the remainder ethanol (EtOH).

Topical treatments

Mice with shaved flanks and tumours measuring ~9 mm^2 held in the i.p. position were treated topically with either 10% TTO/DMSO (50 µl), 10% TTO/acetone, 10% TTO/isopropanol (50 µl), neat TTO (5 µl), 10% TTO-5C/DMSO (50 µl) or vehicle control (10%H_2O/DMSO; 50 µl; five mice per group). Treatments were applied by pipetting the required volume onto the tumour and surrounding skin and rubbing with a gloved finger until the solutions were absorbed through the skin (approx. 20 s). Treatments were applied once daily for 4 days. For topical 5% imiquimod cream (Aldara, INova Pharmaceuticals, NSW, Australia) treatments, ~62.5 mg of the cream was applied to the tumour and surrounding skin once daily for 5 days.

Histology

Mice were euthanised following 1, 2 and 3 topical treatments of either 10% TTO/DMSO or vehicle control. Tumours and surrounding skin, and livers, were fixed in 10% phosphate-buffered formalin overnight, and paraffin wax embedded. Sections were stained with haematoxylin and eosin (H & E) and then examined by a dermatopathologist (PH).

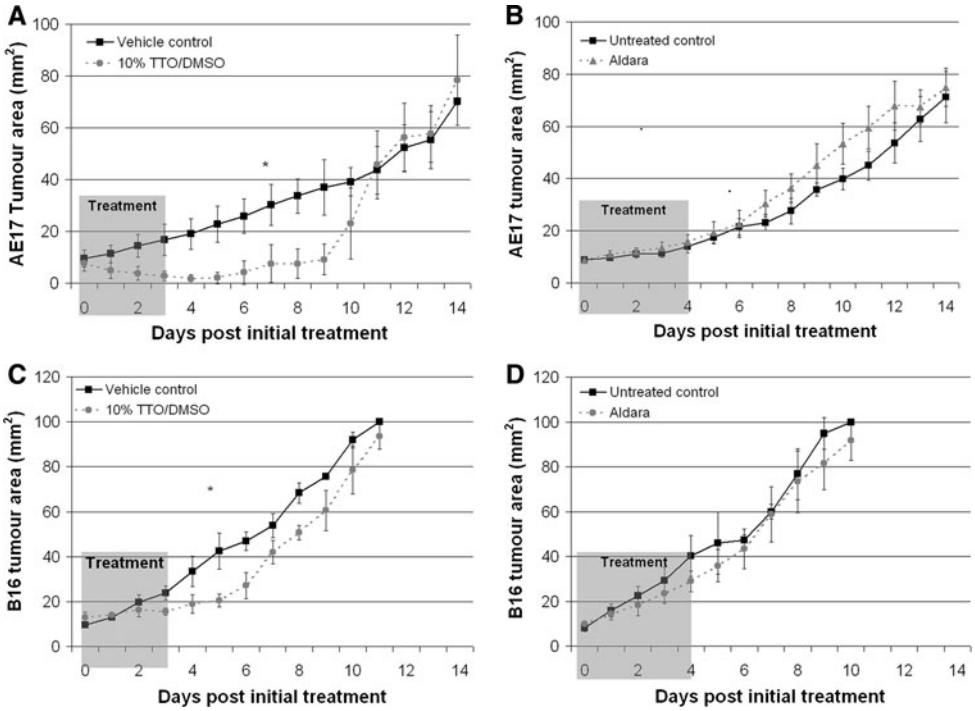

Fig. 1 C57BL/6 J mice with subcutaneous tumours, **a**, **c**: [**a**: AE17 (~9 mm^2), **c**: B16-F10 (~9 mm^2)] treated topically daily for 4 days with 50 µl of 10% TTO/DMSO or 10% H_2O/DMSO (vehicle control). **b**, **d**: [**b**: AE17 (~9 mm^2), **d**: B16-F10 (~9 mm^2)] treated topically daily for 5 days with ~62.5 mg Aldara or left untreated. Data are represented as the mean ±SE of at least four independent topical TTO experiments or at least two independent topical Aldara experiments (five mice per treatment group). *Asterisk* indicate statistically significantly different ($P < 0.05$) from control mice

Statistics

All data are presented as mean ±SE Significant differences as indicated were calculated by Student's *t* tests ($P < 0.05$).

Results

Topical 10% TTO/DMSO induces temporary regression of established subcutaneous AE17 tumours and slows the growth of B16-F10 tumours

Immunocompetent mice with established (~9 mm^2) AE17 subcutaneous tumours were treated topically with 50 µl of 10% TTO/DMSO or 10% H_2O/DMSO alone (vehicle control) for 4 days and compared to untreated controls. Topical 10% TTO/DMSO induced a significant ($P < 0.05$) 10-day period of AE17 tumour growth inhibition compared with vehicle control-treated tumours (Figs. 1a, 2). Moreover, 10% TTO/DMSO treatment induced significant ($P < 0.05$) tumour regression, as tumours on days 2–5 were reduced 2- to 5-fold compared to initial tumour size (day 0; Figs. 1a, 2b). Treatment with 10% H_2O/DMSO had no effect on tumour growth compared with untreated control tumours (Fig. 1a, b). Upon cessation of TTO/DMSO treatment at day 4 (as regulated by AEC due to skin irritation), tumours relapsed but with considerable variability; 2 of 20 mice displayed no signs of relapse until 3 months post treatment. In the remaining 18 mice, tumours resumed control growth levels approximately 4 days after treatment cessation (Fig. 1a). Side effects manifested as skin dryness, erythema and eschar formation in the treated area, and were most severe 2 days post treatment cessation (average skin irritation score 3; Fig. 2b). Skin began to heal 3 days post cessation of treatment and was almost completely resolved by day 10 (Fig. 2b). In addition, the antitumour effect of 10%TTO/DMSO was seen with established B16-F10 melanoma tumours, as treatment significantly ($P < 0.05$) retarded B16-F10 tumour growth in immunocompetent mice (Fig. 1c). However, treated tumours resumed control growth levels 2–3 days following cessation of treatment and grew rapidly to the humane endpoint of 100 mm^2. Topical Aldara applied for 5 days to established tumours, included as a positive control, induced high levels of skin erythema and dryness, yet failed to induce any tumour growth inhibition or regression compared to untreated AE17 or B16-F10 tumours (Fig. 1b, d).

DMSO carrier is required for topical TTO to have antitumour effect in vivo

To demonstrate the importance of using DMSO as a carrier for TTO, we examined the efficacy of 5 µl neat TTO with no carrier (equivalent to 10% TTO in 50 µl DMSO), 10% TTO/acetone and 10% TTO/isopropanol applied topically for 4 days to established AE17 tumours. Neat TTO, 10% TTO in isopropanol or acetone had no inhibitory effect on tumour growth (Fig. 3). Neat TTO induced no skin irritation, whilst both isopropanol and acetone combined with TTO induced minimal local skin irritation which included some dryness and erythema, both of which completely healed upon cessation of treatment.

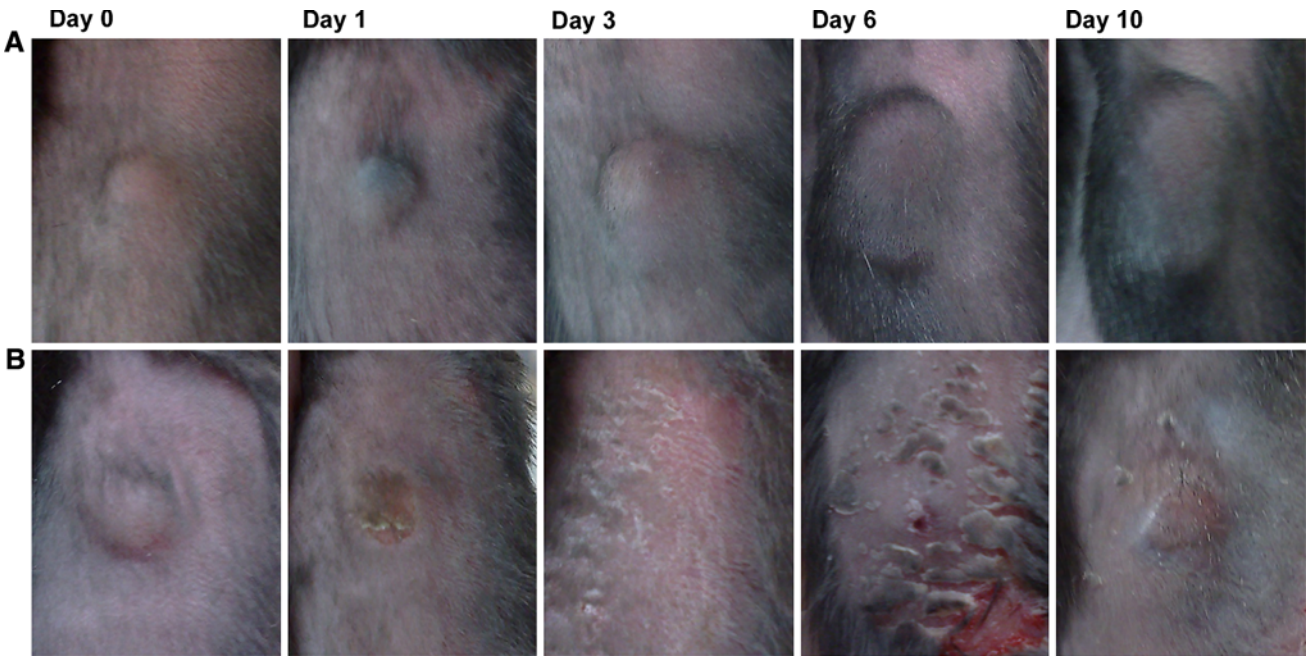

Fig. 2 Representative photographs of C57BL/6 J AE17 tumour-bearing mice, treated with **a** 10% H_2O/DMSO, **b** 10% TTO/DMSO treatments on day 0 (initial treatment), day 1 and 3 (during treatment period), day 6 (3 days post treatment cessation) and day 10 (7 days post treatment cessation). **a** Topical 10% H_2O/DMSO treatment had no effect on tumour growth and induced no skin inflammation, tumour sizes shown on day 0, 1, 3, 6 and 10 were, 9, 12, 20, 36, and 68 mm^2. **b** Topical 10% TTO/DMSO treatment induced tumour growth inhibition and tumour regression with dryness, erythema and eschar formation that was almost completely resolved on day 10, tumour sizes shown on day 0, 1, 3, 6 and 10 were 9, 6, 0, 2 and 20 mm^2 with skin scores on day 0, 1, 3, 6, and 10 of 0, 1, 2, 3, and 1

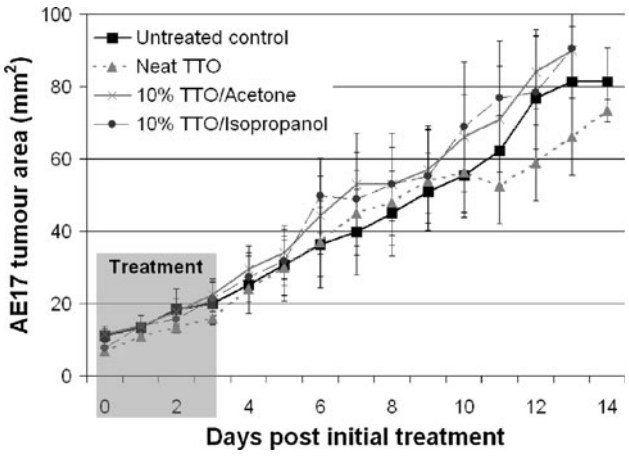

Fig. 3 C57BL/6 J mice with subcutaneous tumours (~9 mm^2AE17) treated topically daily for 4 days with 5 µl Neat TTO, 50 µl of 10% TTO/Acetone or 50 µl of 10% TTO/Isopropanol compared with untreated controls. Data are represented as the mean ±SE of at least two independent experiments (five mice per treatment group)

Combined major components of TTO induce tumour growth inhibition

TTO contains over 100 components. In an effort to identify possible single components or combinations responsible for the antitumour effect of topical TTO, we examined (a) the efficacy of each of the five major components which make up 80% of TTO compared with (b) the combination of the five major components (5C) applied topically together. Doses of the single components were equivalent to those found in 10% TTO i.e. 4% terpinen-4-ol, 2% γ-terpinene, 1% α-terpinene, 0.5% 1,8-cineole and 0.5% ρ-cymene. None of these topically applied major components had any inhibitory effect on established tumours (data not shown). However, the combination (5C) significantly inhibited the growth of established AE17 tumours compared with vehicle control growth (Fig. 4). Specifically, 10% TTO-5C/DMSO applied topically for 4 days significantly ($P < 0.05$) inhibited tumour growth for a period of 5 days (Fig. 4) and induced significant tumour regression in 7 of 15 mice. Tumours resumed control growth levels approximately 2 days following treatment cessation. Side effects included skin dryness, erythema and eschar formation which began to heal 3 days post cessation of treatment and completely resolved by days 10–12.

During the treatment application, 10% TTO/DMSO induces skin irritation that is likely necessary for the antitumour effect and which rapidly resolves upon cessation of treatment

Some local skin irritation resulted from topical application of 10% TTO/DMSO yet no skin reaction was noted following

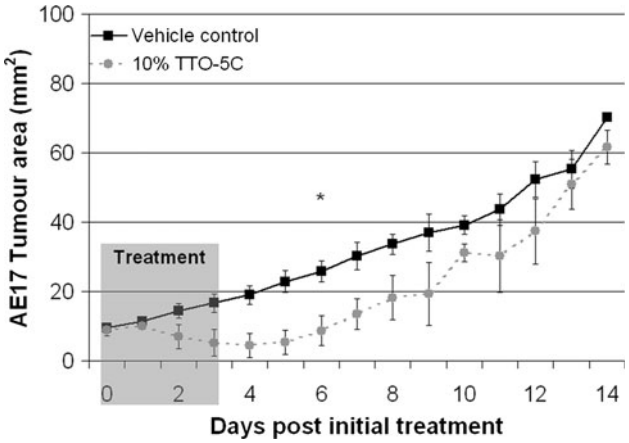

Fig. 4 C57BL/6 J mice with subcutaneous tumours (~9 mm^2 AE17) treated topically daily for 4 days with 50 µl of 10% TTO-5C in DMSO compared with controls. Data are represented as the mean ±SE of at least three independent experiments (five mice per treatment group). *Asterisk* indicate statistically significantly different ($P < 0.05$) from control mice

H_2O/DMSO or neat TTO treatment alone. We observed skin irritation macroscopically which manifested as dryness and erythema following two topical treatments. This progressed to eschar formation at the end of the four treatments which peaked 2 days post treatment cessation and as such limited the treatment to these 4 days (regulated by our AEC). Histological analyses (Fig. 5) showed that epidermal skin irritation was seen only as a result of TTO/DMSO treatments with increased numbers of neutrophils present (Fig. 5b). H_2O/DMSO induced no/little histological evidence of epidermal or dermal inflammation (Fig. 5a). Dermal inflammation was also only seen in TTO/DMSO-treated skin/tumour sections and was again characterised by an abundance of neutrophils and a dense infiltration of more diverse immune cells including macrophages, mast cells and lymphocytes but not eosinophils (Fig. 5b).

Discussion

This study is the first to demonstrate the topical antitumour efficacy of a TTO formulation in immunocompetent, tumour-bearing mice. Four, daily, topical 10% TTO/DMSO treatments of established ~9 mm^2 subcutaneously implanted AE17 mesotheliomas were sufficient to induce a period of significant regression and growth inhibition of these aggressive, chemo-resistant tumours. Although topical treatment with the carrier DMSO alone had no antitumour effect, it was required for TTO-induced tumour eradication.

DMSO is widely used as an efficient penetration enhancer [15, 16] and may act by lowering skin resistance or increasing drug partitioning [17]. Topical treatment with 99% DMSO was successfully used for tissue extravasation reactions following intravenous adriamycin and was well tolerated in human patients [18, 19]. In the current study, mice treated topically with 10% H_2O/DMSO showed no signs of adverse reaction, with normal liver serum enzymes

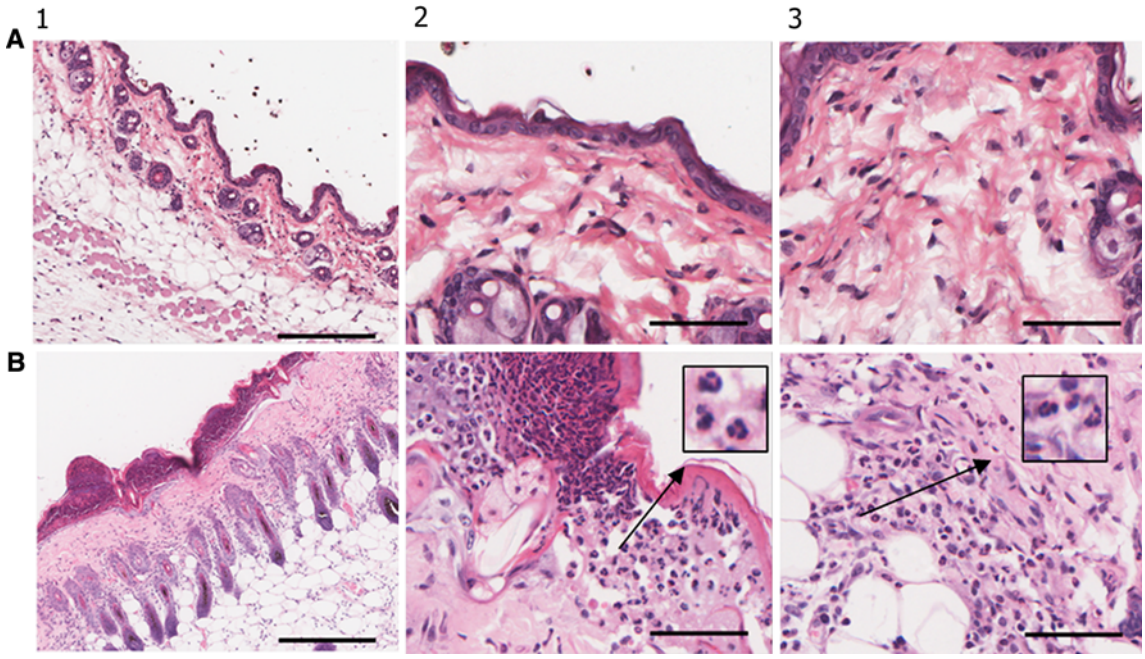

Fig. 5 Representative H & E staining of paraffin embedded sections through the skin of C57BL/6 J mice bearing AE17 tumours (**a**) following 3 topical 10%H_2O/DMSO treatments (vehicle control) (**b**) following 3 topical 10% TTO/DMSO treatments. (*1*) magnification 400×, *scale bar* 50 µm, (*2* and *3*) magnification 1,600×, *scale bar* 12.5 µm. *Inset 2* and *3* to highlight the presence of neutrophils

and liver histology (data not shown), and no apparent ulceration of the skin. We hypothesise that the combination of TTO with DMSO increases the absorption through the skin and decreases the evaporation of TTO, as neat TTO and TTO combined with acetone or isopropanol had no effect in our tumour model.

Studies investigating the penetration of TTO revealed that 1.1–4% TTO penetrates through human epidermis. Over 98% of the oil evaporates within 4 h of its application [20] and only terpinen–4–ol readily penetrates the skin. Formulations to enhance this penetration and reduce evaporation are therefore required. Topical TTO in 5–10% formulations has been used successfully to treat acne, herpes labialis and fungal skin infections (reviewed in [9]). Moreover, TTO has significant anti-inflammatory activity; however, this response maybe dependent on the formulation used. Topically applied neat TTO reduces chemical, histamine- and nickel-induced skin inflammation [21–23], without affecting skin pathology. In addition, a 5% ointment and 10% gel similarly reduced chemically induced skin oedema [24], but a 5% TTO lotion failed to reduce nickel-induced skin oedema [23].

The antitumour effect of topical 10% TTO/DMSO was accompanied by skin irritation which completely resolved on cessation of treatment. Skin irritation following chemotherapeutic agents is typically observed following treatment with topical imiquimod, ingenol-3-angelate and 5-FU [1, 25, 26]. Inflammation is probably required for topical anticancer chemotherapeutic agents to work, as studies of topical imiquimod and 5-FU showed that higher rates of inflammation following treatment were associated with better cancer clearance [26]. Topical TTO/DMSO treatment cessation after 4 treatments was required by our AEC due to the skin inflammation induced. Lower and higher doses of topical TTO/DMSO (3–5% and 15%) applied for longer and shorter treatment periods induced lower and higher levels of skin irritation but failed to induce significant tumour regression in comparison with 10% TTO/DMSO (data not shown). Skin inflammation induced by topical 10% TTO/DMSO resolved upon cessation of treatment; however, the majority of tumours regrew at rates similar to control tumours. In a minority of cases (two mice), complete regression occurred and a period free of macroscopic tumours was observed. The longest of these was 3 months, but in both cases the tumours eventually regrew with kinetics similar to control tumours. Taken together, this further emphasises the potential connection between skin inflammation and the antitumour effect. With the development of a more appropriate formulation, skin inflammation may be reduced and the treatment period could be extended likely decreasing the tumour relapse rate.

Topical TTO/DMSO may act as an adjuvant, similar to topical imiquimod, by the activation of DCs to recruit cytotoxic T cells against skin cancers. Aldara (imiquimod, 5%) is FDA-approved for the clinical treatment of sBCC and actinic keratoses, and is widely recognised as inducing skin inflammation. In our study, TTO combined with acetone or isopropanol had no antitumour effect and induced little skin inflammation, suggesting the importance of skin inflammation to the antitumour outcome. Nonetheless, skin inflammation alone is not sufficient to stimulate an antitumour effect. Doses of Aldara identical to those used in our study induced marked immune cell infiltrate including neutrophils, $CD4^+$ T cells and DCs [25]. Similarly, topical Aldara induced high levels of erythema and skin dryness in our mouse model, yet failed to inhibit AE17 or B16-F10 subcutaneous tumours in immunocompetent mice. Antitumour responses to Aldara are variable [27, 28]. In addition to the skin inflammation, systemic side effects such as headache, flu-like symptoms and long treatment application time of 6 weeks are problematic. The lack of antitumour effect using topical Aldara observed in our tumour-bearing mice indicates that this treatment type may be ineffective against these more aggressive tumour types, as only intratumoural administration of imiquimod retarded the growth of subcutaneous AB1 mesotheliomas [29]. In an additional study, topical imiquimod delayed the growth of subcutaneous B16-F10 tumours, identical to those used in our study, but these tumours were treated immediately post tumour cell inoculation (i.e. prior to the formation of macroscopic tumours) and with a low tumour cell inoculate (2×10^3), and hence with a lower tumour cell burden [30]. The lack of antitumour efficacy of Aldara observed in our studies maybe because of a greater tumour burden with $\sim$9 mm^2 established tumours.

It is feasible that TTO induces other effects on the tumours besides immune activation. Imiquimod induces apoptosis of tumour cells and inhibits tumour vascular formation [31] whilst ingenol-3-angelate also activates protein kinase C and induces primary necrosis of implanted subcutaneous tumours [1]. Melanoma and mesothelioma cells are notoriously resistant to chemotherapy (reviewed in [32] and [33], respectively); moreover, both AE17 and B16 cells failed to undergo apoptosis following staurosporine treatment [8]. Failure of Aldara to induce apoptosis as a result of apoptosis resistance in our aggressive tumour models could also explain the lack of Aldara antitumour effect we observed. TTO induces apoptosis through lipid reorganisation of plasma membranes in human M14 melanoma cells in vitro [7]. Interestingly, our in vitro study highlighted that TTO was more dose effective in AE17 cells and induced low level apoptosis and primary necrotic cell death. Whilst B16-F10 cells were more resistant to either form of cell death, G1 phase arrest was significantly induced following TTO treatment [8]. The differential effect between the two tumour cell types appears to be similar in our in vivo study,

as topical TTO regressed AE17 tumours but only retarded the growth of subcutaneous implanted B16-F10 cells during the treatment period. The variable efficacy in the two tumour models may also be related to the aggressiveness/growth rates of the two tumours. B16-F10 tumours grow very rapidly to reach 100 mm^2 approximately 12 days post tumour cell inoculation, thus having a larger tumour burden for the TTO to act against. Numerous studies have identified that antitumour agents are more effective against small or slower growing tumours [13, 30, 34].

Histological evaluation of TTO/DMSO-treated skin from tumour-bearing mice showed an influx of neutrophils in both the epidermis and the dermis, and the presence of other immune effector cells. This suggests that topical treatment with TTO/DMSO may act by local immune activation. Ingenol-3-angelate induces a neutrophil-mediated antibody-dependent cellular cytotoxicity eradication of residual tumour cells [2]. The role neutrophils play in the antitumor effect of topical TTO needs further investigation, with studies currently underway in our laboratory to characterise the immune cell populations and activation status in the tumour, surrounding skin- and tumour-draining LNs following treatments.

Numerous studies using tumour models have identified that immune activation, specifically, systemically by memory cells, is important for antitumour efficacy and for translation to the clinic [35]. A lasting, memory immune response following treatment is superior to local direct efficacy. TTO may have both direct and indirect effects on the tumours cells.

The antitumour effect of the TTO-5C/DMSO topical treatment, yet lack of effect of the single agents (at concentrations tested), suggests that the effect of topical TTO may be narrowed down to a combination of major active terpenes. In addition, the combination of multiple terpenes may overcome problems such as resistance often seen with single-agent treatment regimes. It is possible that with the right formulation, single-agent TTO terpenes may be as or more effective than TTO as numerous studies of TTO have identified terpinen-4-ol as the major active component that is likely responsible to TTO's efficacy. However, as terpene agents themselves are known to act as penetration enhancers, it is possible that the combination of terpenes enhances the penetration of each other. The lack of systemic activity within the treatment period identified by normal body weight, no changes in serum alkaline phosphatase or aspartate aminotransferase and normal liver histology (data not shown) make TTO or a combination of TTO terpenes ideal candidates for a cost effective, self-administrable skin cancer treatment.

Overall, topical TTO/DMSO represents a potentially safe and effective antitumour treatment. Skin inflammation during the treatment period similar to numerous topical chemotherapeutic agents completely resolves and is probably necessary for TTO's therapeutic effect. The extension of topical TTO treatment time, which may lead to complete cure of tumours and prevention of tumour relapse, could be achieved by the identification of a more suitable TTO formulation. This and studies to further characterise TTO's antitumour mode(s) of action are currently underway.

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References

1. Ogbourne SM, Suhrbier A, Jones B, Cozzi SJ, Boyle GM, Morris M, McAlpine D, Johns J, Scott TM, Sutherland KP, Gardner JM, Le TT, Lenarczyk A, Aylward JH, Parsons PG (2004) Antitumor activity of 3-ingenyl angelate: plasma membrane and mitochondrial disruption and necrotic cell death. Cancer Res 64:2833–2839
2. Challacombe JM, Suhrbier A, Parsons PG, Jones B, Hampson P, Kavanagh D, Rainger GE, Morris M, Lord JM, Le TT, Hoang-Le D, Ogbourne SM (2006) Neutrophils are a key component of the antitumor efficacy of topical chemotherapy with ingenol-3-angelate. J Immunol 177:8123–8132
3. Greg Siller KG, Welburn Peter, Katsamas Janelle, Ogbourne SM (2009) Pep005 (ingenol mebutate) gel, a novel agent for the treatment of actinic keratosis: results of a randomized, double-blind, vehicle-controlled, multicentre, phase iia study. Australas J Dermatol 50:16–22
4. Le TT, Gardner J, Hoang-Le D, Schmidt CW, MacDonald KP, Lambley E, Schroder WA, Ogbourne SM, Suhrbier A (2009) Immunostimulatory cancer chemotherapy using local ingenol-3-angelate and synergy with immunotherapies. Vaccine 27:3053–3062
5. Yeruva L, Pierre KJ, Elegbede A, Wang RC, Carper SW (2007) Perillyl alcohol and perillic acid induced cell cycle arrest and apoptosis in non small cell lung cancer cells. Cancer Lett 257:216–226
6. da Fonseca CO, Linden R, Futuro D, Gattass CR, Quirico-Santos T (2008) Ras pathway activation in gliomas: a strategic target for intranasal administration of perillyl alcohol. Arch Immunol Ther Exp (Warsz) 56:267–276
7. Calcabrini A, Stringaro A, Toccacieli L, Meschini S, Marra M, Colone M, Salvatore G, Mondello F, Arancia G, Molinari A (2004) Terpinen-4-ol, the main component of melaleuca alternifolia (tea tree) oil inhibits the in vitro growth of human melanoma cells. J Invest Dermatol 122:349–360
8. Greay SJ, Ireland DJ, Kissick HT, Levy A, Beilharz MW, Riley TV, Carson CF (2009) Induction of necrosis and cell cycle arrest in murine cancer cell lines by melaleuca alternifolia (tea tree) oil and terpinen-4-ol. Cancer Chemother Pharmacol. doi:10.1007/s00280-009-1093-7
9. Carson CF, Hammer KA, Riley TV (2006) Melaleuca alternifolia (tea tree) oil: a review of antimicrobial and other medicinal properties. Clin Microbiol Rev 19:50–62
10. Needham DJ, Lee JX, Beilharz MW (2006) Intra-tumoural regulatoryt cells: a potential new target in cancer immunotherapy. Biochem Biophys Res Commun 343:684–691
11. Kissick HT, Ireland DJ, Beilharz MW (2008) Combined intratumoural regulatory t-cell depletion and transforming growth factor-β neutralisation induces regression of established ae17 murine mesothelioma tumours. J Interferon Cytokine Res 29:1–8

12. Currie AJ, van der Most RG, Broomfield SA, Prosser AC, Tovey MG, Robinson BWS (2008) Targeting the effector site with ifn-{alpha}{beta}-inducing tlr ligands reactivates tumor-resident cd8 t cell responses to eradicate established solid tumors. J Immunol 180:1535–1544
13. Jackaman C, Bundell CS, Kinnear BF, Smith AM, Filion P, van Hagen D, Robinson BW, Nelson DJ (2003) Il-2 intratumoral immunotherapy enhances cd8 + t cells that mediate destruction of tumor cells and tumor-associated vasculature: a novel mechanism for il-2. J Immunol 171:5051–5063
14. I.O.f. Standardisation (2004) Iso 4730:2004: oil of melaleuca, terpinen-4-ol type (tea tree oil). International Organisation for Standardisation, Geneva
15. Williams AC, Barry BW (2004) Penetration enhancers. Adv Drug Deliv Rev 56:603–618
16. Gurtovenko AA, Anwar J (2007) Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. J Phys Chem B 111:10453–10460
17. Walker RB, Smith EW (1996) The role of percutaneous penetration enhancers. Adv Drug Deliv Rev 18:295–301
18. Lopez AM, Wallace L, Dorr RT, Koff M, Hersh EM, Alberts DS (1999) Topical dmso treatment for pegylated liposomal doxorubicin-induced palmar-plantar erythrodysesthesia. Cancer Chemother Pharmacol 44:303–306
19. Bertelli G, Gozza A, Forno GB, Vidili MG, Silvestro S, Venturini M, Del Mastro L, Garrone O, Rosso R, Dini D (1995) Topical dimethylsulfoxide for the prevention of soft tissue injury after extravasation of vesicant cytotoxic drugs: a prospective clinical study. J Clin Oncol 13:2851–2855
20. Cross SE, Russell M, Southwell I, Roberts MS (2008) Human skin penetration of the major components of australian tea tree oil applied in its pure form and as a 20% solution in vitro. Eur J Pharm Biopharm 69:214–222
21. Khalil Z, Pearce AL, Satkunanathan N, Storer E, Finlay-Jones JJ, Hart PH (2004) Regulation of wheal and flare by tea tree oil: complementary human and rodent studies. J Invest Dermatol 123:683–690
22. Brand C, Townley SL, Finlay-Jones JJ, Hart PH (2002) Tea tree oil reduces histamine-induced oedema in murine ears. Inflamm Res 51:283–289
23. Pearce AL, Finlay-Jones JJ, Hart PH (2005) Reduction of nickel-induced contact hypersensitivity reactions by topical tea tree oil in humans. Inflamm Res 54:22–30
24. Brand C, Grimbaldeston MA, Gamble JR, Drew J, Finlay-Jones JJ, Hart PH (2002) Tea tree oil reduces the swelling associated with the efferent phase of a contact hypersensitivity response. Inflamm Res 51:236–244
25. van der Fits L, Mourits S, Voerman JSA, Kant M, Boon L, Laman JD, Cornelissen F, Mus A-M, Florencia E, Prens EP, Lubberts E (2009) Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the il-23/il-17 axis. J Immunol 182:5836–5845
26. Jury CS, Ramraka-Jones VS, Gudi V, Herd RM (2005) A randomized trial of topical 5% 5-fluorouracil cream in the treatment of actinic keratoses comparing daily with weekly treatment. Br J Dermatol 153:808–810
27. Sidky YA, Borden EC, Weeks CE, Reiter MJ, Hatcher JF, Bryan GT (1992) Inhibition of murine tumor growth by an interferon-inducing imidazoquinolinamine. Cancer Res 52:3528–3533
28. Peter KL, William BH, Keith HL, Tania JP, David AW, Kara LA, James HL (2005) Long-term clinical outcomes following treatment of actinic keratosis with imiquimod 5% cream. Dermatol Surg 31:659–664
29. Broomfield SA, van der Most RG, Prosser AC, Mahendran S, Tovey MG, Smyth MJ, Robinson BW, Currie AJ (2009) Locally administered tlr7 agonists drive systemic antitumor immune responses that are enhanced by anti-cd40 immunotherapy. J Immunol 182:5217–5224
30. Lee JR, Shin JH, Park JH, Song SU, Choi GS (2007) Combined treatment with intratumoral injection of dendritic cells and topical application of imiquimod for murine melanoma. Clin Exp Dermatol 32:541–549
31. Sidbury R, Neuschler N, Neuschler E, Sun P, Wang XQ, Miller R, Tomai M, Puscasiu E, Gugneja S, Paller AS (2003) Topically applied imiquimod inhibits vascular tumor growth in vivo. J Invest Dermatol 121:1205–1209
32. Grossman D, Altieri DC (2001) Drug resistance in melanoma: Mechanisms, apoptosis, and new potential therapeutic targets. Cancer Metastasis Rev 20:3–11
33. Fennell DA, Rudd RM (2004) Defective core-apoptosis signalling in diffuse malignant pleural mesothelioma: Opportunities for effective drug development. Lancet Oncol 5:354–362
34. DeLong P, Tanaka T, Kruklitis R, Henry AC, Kapoor V, Kaiser LR, Sterman DH, Albelda SM (2003) Use of cyclooxygenase-2 inhibition to enhance the efficacy of immunotherapy. Cancer Res 63:7845–7852
35. Darrasse-Jaze G, Bergot AS, Durgeau A, Billiard F, Salomon BL, Cohen JL, Bellier B, Podsypanina K, Klatzmann D (2009) Tumor emergence is sensed by self-specific CD44hi memory Tregs that create a dominant tolerogenic environment for tumors in mice. J Clin Invest 119:2648–2662