Terpinen-4-ol, The Main Component of *Melaleuca Alternifolia* (Tea Tree) Oil Inhibits the *In Vitro* Growth of Human Melanoma Cells

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The search for innovative therapeutic approaches based on the use of new substances is gaining more interest in clinical oncology. In this *in vitro* study the potential anti-tumoral activity of tea tree oil, distilled from *Melaleuca alternifolia*, was analyzed against human melanoma M14 WT cells and their drug-resistant counterparts, M14 adriamicin-resistant cells. Both sensitive and resistant cells were grown in the presence of tea tree oil at concentrations ranging from 0.005 to 0.03%. Both the complex oil (tea tree oil) and its main active component terpinen-4-ol were able to induce caspase-dependent apoptosis of melanoma cells and this effect was more evident in the resistant variant cell population. Freeze-fracturing and scanning electron microscopy analyses suggested that the effect of the crude oil and of the terpinen-4-ol was mediated by their interaction with plasma membrane and subsequent reorganization of membrane lipids. In conclusion, tea tree oil and terpinen-4-ol are able to impair the growth of human M14 melanoma cells and appear to be more effective on their resistant variants, which express high levels of P-glycoprotein in the plasma membrane, overcoming resistance to caspase-dependent apoptosis exerted by P-glycoprotein-positive tumor cells.

Key words: tea tree oil/melanoma cells/multidrug resistance.


Cutaneous melanoma is a highly invasive and metastatic tumor, highly refractory to chemotherapy. Melanoma cells are known to exhibit both *in vitro* and *in vivo* a high level of intrinsic resistance to various cytostatic agents (Schadendorf et al., 1994). Moreover, following drug treatment, melanoma cells can acquire the classical multidrug resistance (MDR) phenotype, characterized by reduced intracellular drug accumulation and high resistance index. Several studies suggest that a class of specific drug-transporter proteins, including P-glycoprotein (P-gp) (Berger et al., 1994; Alvarez et al., 1995; Molinari et al., 1998, 2000) and MDR-related protein (MRP1) (Cole et al., 1992), might play a central part in the establishment of the MDR phenotype in melanoma cells. Several *in vitro* and *in vivo* studies have been performed as an attempt to reverse the drug resistance phenotype and to develop innovative chemotherapeutic strategies effective against MDR tumors. Most of the MDR modulators, such as calcium channel blockers (e.g., verapamil), calmodulin inhibitors (e.g., trifluoperazine), and immunosuppressive agents (e.g., cyclosporine A, CsA) (Sikic, 1993) induce a competitive inhibition of the P-gp molecule activity. Unfortunately, the use of these substances *in vivo* presents plenty of obstacles due to a number of factors: (1) inhibitory concentrations of modulators are very toxic to patients (hypotension, heart block, myelosuppression); (2) bioavailability decreases as these modulating compounds are bound to certain macromolecules such as serum proteins; (3) hematologic malignancies appear to be more sensitive to MDR modulators than solid tumors, where different mechanisms are responsible for the MDR phenotype. Therefore, the search for innovative therapeutic approaches, based on the use of new substances effective against resistant tumors, is gaining more and more interest in clinical oncology.

Tea tree oil (TTO) is an aboriginal Australian traditional medicine for bruises, insect bites, and skin infections. It was rediscovered in the 1920s as a topical antiseptic with more effective activity than phenol. TTO is the essential oil steam distilled from *Melaleuca alternifolia*, a species of northern New South Wales, Australia. The oil is considered non-poisonous, it possesses a pleasant odor and it is included in a large range of products for skin and wound care. In fact, the lipophilic nature of the oil that enables it to penetrate the skin, suggests it may be suitable for topical therapeutic use in the treatment of fungal mucosal and cutaneous infections. The chemical composition of TTO has been well defined and consists of a complex mixture of monoterpenoids of which about 50% are oxygenated and about 50% are hydrocarbons (Brophy et al., 1989). The active component of the oil is thought to be terpinen-4-ol, although synergistic effects from other terpenes cannot be excluded (Altman, 1988). TTO exhibits broad-spectrum anti-microbial activity (Markham, 1999), which can be principally attributed to terpinen-4-ol (Southwell et al., 1993; Carson and Riley, 1995) and there are susceptibility data on a wide range of gram-positive and negative bacteria (Altman, 1988; Carson...
et al., 1998; Anderson and Fennessy, 2000; Caelli et al., 2000; May et al., 2000; Zhang and Robertson, 2000). Moreover, the anti-fungal activity of TTO against yeasts and dermatophytes is reported in several papers (Nenoff et al., 1996; Syed et al., 1999; Ernst and Huntley, 2000; D’Auria et al., 2001). TTO has been used in the treatment of acne (Basset et al., 1990) and Tinea pedis (Tong et al., 1992). Finally, several studies investigated the potential anti-inflammatory properties of TTO and recent data demonstrated that terpinen-4-ol suppresses inflammatory mediator production by activated human monocytes (Hart et al., 2000).

In this in vitro study the potential anti-tumoral activity of TTO was analyzed against human melanoma M14 wild type (WT) cells and their drug-resistant counterparts, M14 adriamicin-resistant (ADR) cells, selected by prolonged exposure to doxorubicin (DOX). Results herein reported demonstrate that both TTO and its main component terpinen-4-ol are able to impair the growth of human M14 melanoma cells and appear to be more effective on the drug-selected resistant cell line M14 ADR, which express high levels of P-gp in the plasma membrane.

**Results**

Selection and characterization of resistant melanoma cells Human melanoma M14 WT cells were cultured in the presence of the anti-tumoral drug DOX, an anthracyclinic antibiotic largely employed in anti-neoplastic regimens. A drug-resistant cell line (M14 ADR) was selected after numerous passages (≈ 100) in the presence of 40 μM DOX.

In order to evaluate the drug sensitivity of M14 ADR cells, a cloning efficiency assay was carried out. Resistant cells showed a remarkable decrease of sensitivity to DOX with a resistance index of 48. The acquired resistance to the anti-tumoral drug matched the appearance of P-gp on the plasma membrane (Fig 1a,b), as revealed by flow cytometric analysis. The expression of P-gp was confirmed by western blotting (Fig 1c) and reverse transcription–PCR experiments (Fig 1d). The low expression of P-gp in drug-sensitive M14 cells was not detected by western blot technique. On the contrary, in agreement with previous studies performed by our group (Molinari et al., 1998), reverse transcription–PCR of M14 WT cells demonstrated the presence of the mRNA of MDR1 gene.

Chemical identification and quantitative estimation of TTO Chemical components were identified by comparing their GC retention times, the Kovat’s Indices (Adams, 1995) and GC/MS spectra with those of the reference substances.

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**Figure 1**

Expression of P-gp on human melanoma M14 sensitive and drug-selected cells. (a,b) Flow cytometric determination of surface P-gp in human melanoma M14 WT (a) and M14 ADR (b) cells labeled with MoAb MM4.17 (black histograms) or mouse isotypic globulins (negative control, dotted histograms). (c) Western blot of cell lysates from M14 WT (1) and M14 ADR (2) cells. The membrane was probed with the primary MoAb to P-gp (MoAb C219) and to actin (MoAb 1501). (d) Detection of MDR1 mRNA. PCR amplification products (40 cycles) fractioned by 2% agarose gel and visualized by ethidium bromide for human breast carcinoma MCF7-DX (positive control) (2), human melanoma M14 WT (3), and M14 ADR (4) cells. (1) DNA marker.

Quantitative data were based on peak area normalization without using a correction factor.

The oil contained 42.35% terpinen-4-ol, 20.65% γ-terpinene, 9.76% α-terpinene, 3.71% terpinolene, 3.57% 1,8 cineole, 3.09% α-terpineol, 2.82% p-cimene, 2.42%...
a-pinene, 1.75% limonene, 1.05 δ-cadinene, 0.94% α-thujene, 0.94% aromadendrene, 0.87% myrcene, 0.73% β-pinene, 0.40% sabinene, and 0.34% α-phellandrene. The oil was observed to be of terpinen-4-ol type according to International Standard ISO 4730: 1996.

Effects of TTO on cell growth

In this study, human melanoma M14 WT and M14 ADR cells were grown in the presence of TTO at concentrations ranging from 0.005 to 0.03%. Changes in the cell pool size during the exposure to different oil concentrations are shown as growth curves (Fig 2a,b). At the lowest concentration (0.005%), no difference in cell growth could be revealed between control and TTO-treated cells, both in parental and resistant cell lines. The presence of 0.01% TTO did not affect the growth of M14 WT cells, whereas it induced a slight, but significant, decrease of the cell pool size in the resistant variant. The two higher concentrations (0.02, 0.03%) were strongly inhibitory for the growth of both parental M14 WT and resistant M14 ADR cells. The effect proved to be more pronounced in the M14 ADR cell line than in M14 WT one, however.

Induction of cell apoptosis

Apoptosis as revealed by the annexin V binding method (Vermes et al, 1995) was seen under treatment with TTO ranging from 0.005 to 0.02% for 48, 72, and 96 h. After 72 and 96 h of treatment with 0.02% TTO, an increased number of necrotic cells (positive for both annexin V and PI) was revealed, which impaired a correct AI determination. As shown in Fig 3 the maximum AI was reached under the treatment with 0.02% TTO for 48 h both for M14 WT and M14 ADR cells. After 72 and 96 h of treatment with 0.02% TTO, an increased number of necrotic cells (positive for both annexin V and PI) was revealed, which impaired a correct AI determination. It is interesting to note that the AI values detected in treated samples were generally greater in the resistant variant cell population than in the parental one.

Noteworthy, M14-resistant cells appeared to be less sensitive to caspase-dependent apoptotic stimuli, such as serum starvation (Fig 4) or anti-Fas MoAb-induced apoptosis (Fig 5), even if they expressed equivalent levels of surface Fas as determined by flow cytometry (data not shown).

There was a negative correlation between the AI and the cell pool size. In Table I the negative exponential correlations between cell pool size and AI of M14 WT and M14 ADR melanoma cells after 48, 72, and 96 h of treatment are reported.

The active component of the oil is thought to be terpinen-4-ol (Altman, 1988); thus, to analyze its contribution to the induction of apoptosis, experiments performed with 0.01% pure terpinen-4-ol for 48 h were carried out. The choice of terpinen-4-ol concentration was determined by the results of the chemical identification and quantitative estimation of TTO used in this work (42.35% terpinen-4-ol). Interestingly, as evaluated by measuring DNA fragmentation with TUNEL assay, also terpinen-4-ol alone induced a higher percentage of apoptotic cells in the resistant M14 ADR cell population, when compared with the sensitive one (Fig 6). Moreover, in order to gain insight into the involvement of P-gp in the response of M14 cells to the induction of apoptosis by terpinen-4-ol, the treatment was performed in the absence or in the presence of CsA, at the concentration (5 μM) that proved to be able to inhibit the transport function of P-gp (Molinari et al, 2000). The inhibition of the MDR transporter did increase noticeably the percentage of apoptotic cells in the M14 WT cell line and to a lesser extent in M14 ADR cells.

Furthermore, both M14 WT and M14 ADR cells were treated with 0.02% TTO or 0.01% terpinen-4-ol for 48 h, in the absence or in the presence of caspase inhibitors (Fig 7a,c). To inhibit the activation of caspasess, cells were pretreated for 60 min with peptidyl fluoromethylketones. Both z-DEVD-fmk (caspase-3 inhibitor) and z-VAD-fmk (broad-spectrum caspase inhibitor) (20 μM) significantly decreased the percentage of TUNEL-positive cells in both sensitive and resistant cell cultures. Such results indicated that DNA fragmentation was dependent on caspase activation. Experiments of cell viability, based on the Trypan...
blue exclusion method, were also performed (Fig 7b,d). The presence of caspase inhibitors did not influence the percentage of cells able to extrude the dye in M14 WT cultures treated with terpinen-4-ol. By contrast, both in M14 WT cells treated with TTO, and in M14 ADR cells treated with terpinen-4-ol, an increase of about 12% of the number of viable cells was detected. Finally, in the resistant cell population, treatment with TTO in the presence of z-DEVD-fmk or z-VAD-fmk induced an increase of viability of about 23%. In this last case, the presence of a percentage of dead cells following other death pathways cannot be ruled out. Control z-FA-fmk inhibitor (a chymase inhibitor) did not impair TTO (data not shown) and terpinen-4-ol apoptotic death (Fig 7c).

Electron microscopy analysis To get a better insight into the effects of TTO on human melanoma cells, SEM and FF analyses were performed on control and treated M14 cells. SEM observations revealed a concentration-dependent effect of the plant extract on cell surface morphology (Figs 8 and 9). Control M14 WT and M14 ADR cells displayed elongated bipolar or polygonal cells, with the surface covered by randomly distributed microvilli (Figs 8a and 9a, respectively). After treatment for 48 h with the lowest dose (0.005%) of TTO, both parental and resistant cells showed a surface morphology similar to untreated cells (Figs 8b and 9b, respectively). The treatment for 48 h with 0.01% TTO did induce significant morphologic changes in both M14 WT and M14 ADR cells (Figs 8c and 9c, respectively); however, rounded and suffering cells were detectable more frequently in the resistant cell cultures (Fig 9c, arrow) than in the sensitive ones. Finally, 0.02% TTO 48 h-treated cells displayed evident changes in the cell shape...
Table I. Negative exponential correlations between cell pool size and AI of M14 WT and M14 ADR melanoma cells after 48, 72, and 96 h of treatment with TTO

<table>
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<tr>
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<th>M14 WT</th>
<th>M14 ADR</th>
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<tr>
<td>48 h</td>
<td>y = 64.761 e^{(-4.8062x-0.05x)}</td>
<td>y = 63.007 e^{-7.5714e-0.05x}</td>
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<tr>
<td>R</td>
<td>0.98548</td>
<td>0.99639</td>
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<tr>
<td>72 h</td>
<td>y = 38.095 e^{(-1.8395-0.05x)}</td>
<td>y = 45.083 e^{(-2.6666e-0.05x)}</td>
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<tr>
<td>R</td>
<td>0.71361</td>
<td>0.91832</td>
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<tr>
<td>96 h</td>
<td>y = 32.179 e^{(-5.3579e-0.06x)}</td>
<td>y = 19.759 e^{(-9.0424e-10x)}</td>
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<tr>
<td>R</td>
<td>0.94585</td>
<td>0.9885</td>
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Figure 6
Apoptosis induced in sensitive (M14 WT) and resistant (M14 ADR) melanoma cells by treatment with terpinen-4-ol in the presence or absence of CsA. Mean values and SD from three different experiments.

and alterations of the surface morphology with loss of microvilli and the formation of numerous blebs. These modifications were detectable on most M14 WT cells (Fig 8d) and appeared to be even more evident on resistant M14 ADR cells (Fig 9d).

Similar morphologic changes were detected in M14 WT and M14 ADR cell cultures treated with 0.01% terpinen-4-ol for 48 h (Fig 10). A higher number of detaching and blebbing cells were observed in treated resistant cell cultures (Fig 10c,d) when compared with sensitive ones (Fig 10a,b).

Noteworthy, typical alterations of plasma membrane were detectable in terpinen-4-ol-treated cells (Fig 10d), visible more frequently in M14 ADR cells.

Moreover, in order to analyze the possible effects of TTO on the molecular organization of the plasma membrane of human melanoma cells, sensitive and resistant M14 cells were also examined by FF, before and after treatment with 0.02% TTO for 48 h. In control M14 WT and M14 ADR cells, protein intramembrane particles appeared randomly distributed on both exoplasmic (Fig 11a) and protoplasmic (Fig 11b) fracture faces of the plasma membrane. After treatment with TTO, morphologic alterations typical of apoptosis were detected, particularly on resistant M14 ADR cells (Fig 12). In fact, in agreement with SEM observations, numerous blebs protruding from the cell surface were observed (Fig 12a). Membrane blebs displayed both clustered intramembrane particles (Fig 12b) and smooth lipid areas (Fig 12c). In addition, in treated cells globular lipid domains of about 200 to 250 nm in diameter were revealed; this phenomenon was particularly evident on the protoplasmic fracture face (Fig 13) where the intramembrane particles are more numerous. These smooth lipid domains tended to extrude from the plasma membrane (Fig 13b) suggesting a reorganization of membrane lipids induced by TTO.

Discussion
A drug-resistant cell line (M14 ADR) was selected after numerous passages (≈ 100) in the presence of 40 μM DOX. The low expression of P-gp in drug-sensitive M14 WT cells was not detected by western blot technique. On the contrary, in agreement with previous studies performed by our group (Molinari et al., 1998), reverse transcription-PCR of M14 WT cells demonstrated the presence of the mRNA of MDR1 gene. In fact, in established human melanoma cell lines, such as M14, H14, and JR8, which never underwent cytotoxic drug treatment, P-gp was absent on the plasma membrane but it was clearly expressed in the Golgi apparatus, thus accounting for the intrinsic resistance of human melanoma. The exposure to DOX induced the overexpression of the total MDR transporter and its appearance on the plasma membrane, as already observed in other drug-selected M14 cell lines (Molinari et al., 2000).

In a previous paper the cytotoxic effects of TTO were analyzed on human epithelial and fibroblast cells at concentrations ranging from 10 to 1000 μg per mL (Söderberg et al., 1996). The cell lines employed in that study were chosen as they have similar susceptibilities as basal keratinocytes, to topical agents (Teepe et al., 1993). The authors reported that concentrations of TTO lower than 300 μg per mL (≈ 0.03%) were not cytotoxic for the tested lines. In this study human melanoma M14 WT and M14 ADR cells were grown in the presence of TTO at the concentrations ranging from 0.005 to 0.03%. The two higher concentrations (0.02% and 0.03%) appeared to be strongly inhibitory for both the growth of both parental M14 WT and resistant M14 ADR cells; however, the effect proved to be more pronounced in the M14 ADR cell line than in the M14 WT one.

The analysis performed by the annexin V binding method revealed that the treatment with TTO induced the increase of apoptotic cell number in both M14 WT and M14 ADR cell populations. It has been shown that loss of phospholipid asymmetry of the plasma membrane is an early event of apoptosis. The annexin V binds to negatively charged phospholipids, such as phosphatidylserine. During apoptosis the cells react to annexin V as soon as chromatin condenses but before the plasma membrane loses its ability to exclude dyes such as PI (Vermes et al., 1995). Noteworthy, the AI values detected in treated samples were generally greater in the resistant variant cell population than in the parental one. Moreover, there was a negative correlation between the AI and the cell pool size. These
correlations might by themselves indicate that the cell pool size of treated M14 cultures, when compared with untreated ones, is primarily determined by apoptosis, i.e., that the interaction with TTO stimulates programmed cell death in melanoma cells. As the active component of the oil is thought to be terpinen-4-ol (Altman, 1988), additional experiments were performed by employing this component at the concentration close to that detected in the employed crude oil mixture (about 50%). Data obtained from terpinen-4-ol experiments clearly confirmed the higher susceptibility of resistant cells to this component when compared with the sensitive ones.

Previous data demonstrated that functional P-gp can confer resistance to a wide range of caspase-dependent apoptotic stimuli, such as ligation of cell surface death receptors, serum starvation, and ultraviolet irradiation (Robinson et al., 1997; Smyth et al., 1998). In particular, functional P-gp inhibited activation of caspase-3 following Fas ligation and this inhibitory effect could be reversed using P-gp antagonists such as specific anti-P-gp MoAb or

Figure 7
Percent apoptosis (a,c) and cell survival (b,d) in sensitive (M14 WT) and resistant (M14 ADR) melanoma cells by treatment with TTO (a,b) or terpinen-4-ol (c,d) in the presence or absence of caspase inhibitors. Mean values and SD from three different experiments.

Figure 8
Scanning electron microscopy of human melanoma M14 WT cell cultures. (a) Control cells. Cells treated with (b) 0.005% TTO, (c) 0.01% TTO, and (d) 0.02% TTO.
pharmacologic inhibitors (Smyth et al, 1998). Many chemotherapeutic drugs, such as doxorubicin and vincristine, function in a caspase-dependent manner (Smyth et al, 1998; Johnstone et al, 1999); therefore, P-gp may play a dual role in regulating cell death induced by these stimuli (1) by removing the toxins from the cells, and (2) by inhibiting the activation of caspases. Importantly, P-gp does not offer cell protection from death induced by lytic concentrations of the pore-forming protein perforin (Johnstone et al, 1999) or by combination of granzyme B and sublytic concentrations of perforin (Smyth et al, 1998; Johnstone et al, 1999), which together can function in a caspase-independent manner (Johnstone et al, 1999; Smyth et al, 1998; Trapani et al, 1998). At least two molecular pathways leading to caspase-dependent apoptosis have been defined. The best-defined pathway involves ligation of death receptors, typically members of the tumor necrosis factor superfamily such as Fas and tumor necrosis factor receptor, at the cell surface resulting in sequential activation of proximal caspase-8 and downstream effector caspases such as caspase-3 (Boldin et al, 1996; Muzio et al, 1996; Srinivasula et al, 1996; Walczak and Krammer, 2000). The second caspase-dependent pathway involves disruption of the mitochondrial transmembrane potential and the release of mitochondrial

Figure 9
Scanning electron microscopy of human melanoma M14 ADR cell cultures. (a) Control cells. Cells treated with (b) 0.005% TTO, (c) 0.01% TTO, and (d) 0.02% TTO.

Figure 10
Scanning electron microscopy of human melanoma M14 WT (a,b) and M14 ADR cells (c,d) treated with 0.01% terpinen-4-ol for 48 h.
proteins such as cytochrome c (Liu et al., 1996; Kluck et al., 1997; Nagata, 2000) and apoptosis-inducing factors (Susin et al., 1999).

Results obtained in this study clearly demonstrated that P-gp does not protect against TTO- or terpinen-4-ol-stimulated apoptosis. Indeed, P-gp-positive (M14 ADR) cells showed to be more susceptible to TTO- or terpinen-4-ol-action. Moreover, a role of P-gp in the induction of apoptosis seems to be ruled out by the results of the experiments performed by using the combined treatment of the oil with CsA. In fact, CsA, the well known P-gp modulator agent (Saeki et al., 1993) did increase remarkably the number of apoptotic cells mainly in wild-type melanoma cells, which express low levels of the drug transporter. In resistant cells the effects of terpinen-4-ol and CsA appeared to be simply additive in the combined treatment. As P-gp-positive cells remain sensitive to caspase-independent death, it can be argued that TTO and its active component induce apoptosis through this pathway. Note-worthy, experiments performed in the presence of caspase inhibitors (z-VAD-fmk, Z-DEVD-fmk) indicated that TTO and terpinen-4-ol induced a caspase-dependent form of apoptosis, overcoming P-gp protection. Preliminary results obtained by JC1 staining (data not shown) indicated a depolarization of mitochondria after treatment with both the oil and the active component suggesting that the activation of caspase-3 could result from disruption of mitochondrial membrane potential, release of cytochrome c, and subsequent activation of caspase 9.

The effect of TTO on the plasma membrane has been previously hypothesized as terpenes are lipophilic and partition into the phospholipid layer of cell membranes, disrupting normal structure and function (Sikkema et al., 1995). Although the in vitro anti-microbial activity and in vivo efficacy of TTO have been reported, less is known about its mechanism of action. TTO is well characterized and contains approximately 100 terpenes and their related alcohols (Brophy et al., 1989). It is well known that cyclic hydrocarbons, such as aromatics, alicycles, and terpenes, interact with biologic membranes (Uribe et al., 1985). These interactions lead to changes in structure and function of the membranes, which in turn, may impair growth and activity
of the cells (Sikkema et al, 1992). Toxic effects on membrane structure and function have generally been used to explain the anti-microbial action of essential oils and their monoterpenoid components (Uribe et al, 1985; Knobloch et al, 1988). Sikkema et al (1994) showed that, as a result of their lipophilic character, cyclic hydrocarbons will preferentially partition from an aqueous phase into membrane structures. This results in membrane expansion, increased membrane fluidity and inhibition of membrane-embedded enzymes. Consequently, the interaction with the hydrophobic structures of bacteria play a key role in the anti-microbial action of hydrocarbons (Sikkema et al, 1995). Cox et al (2000) confirmed that the anti-microbial activity of TTO results from its ability to disrupt the permeability barrier of microbial membrane structures. In fact, they observed an increased cell permeability to the fluorescent nucleic acid stain PI. Similarly, Carson et al, (2002) reported that TTO and its components compromise the cytoplasmic membranes of Staphylococcus aureus.

Söderberg et al (1996) hypothesized that the cytotoxic activity of TTO on human epithelial and fibroblastic cells is probably exerted by membrane-associated reactions. In our study, results obtained by SEM and FF experiments strongly suggested a preferential interaction of both TTO and terpinen-4-ol with the membranes of resistant M14 cells. MDR is usually mediated by overexpression of P-gp; a large body of work indicates that MDR is also associated with marked changes in membrane lipid composition (Lavie et al, 1999; Santini et al, 2001). In particular, elevated levels of cholesterol, glycosphingolipids, and sphingomyelin have been reported. The greater sensitivity to the TTO treatment displayed by M14 ADR cells when compared with parental ones could be due to different lipid composition of the plasma membrane.

Our results clearly demonstrated that TTO and terpinen-4-ol are able to interfere with the growth of human melanoma cells stimulating programmed cell death as evaluated by annexin V binding experiments, which exclude PI stained cells, and in situ DNA fragmentation labeling. On the other hand, the transbilayer movement and breakdown of sphingomyelin during apoptosis cause concomitant cholesterol efflux and, thus, significant alterations in the biophysical properties of the plasma membrane, which is a prerequisite for membrane blebbing and vesiculation at the surface of the apoptotic cell (Tepper et al, 2000). TTO and terpinen-4-ol, at concentrations lower than 0.03%, perturb molecular architecture of the plasma membrane without completely disrupting it, thus stimulating programmed cell death.

The majority of current anti-cancer therapies induce tumor cell death through the induction of apoptosis. Alterations in the apoptotic pathways may determine tumor resistance to these therapies (Coultas and Strasser, 2000). Activation of the proteolytic cascade involving caspase family members is a critical component of the execution of cell death in apoptotic cells. Recent studies, however, suggest that cell death can proceed in the absence of caspases (Kolenko et al, 2000).

Further studies are now in progress to explore the pathway of TTO or terpinen-4-ol-induced programmed cell death. It is noteworthy that these compounds are apparently able to overcome P-gp-mediated resistance to the caspase-dependent form of apoptosis. A better understanding of the mechanism of action of TTO and of the diverse modes of tumor cell death will help to avoid ineffective anti-tumor treatments. Moreover, TTO might offer promising applications in combined therapy with classical cytotoxic agents, in particular against drug-resistant melanomas.

**Material and Methods**

**Cell cultures** The established human melanoma cell line (M14 WT) and its derivative MDR variant (M14 ADR) were grown in RPMI 1640 medium (Flow Laboratories, Irvine, UK) supplemented with 1% nonessential amino acids, 1% L-glutamine, 100 IU per mL penicillin, 100 IU per mL streptomycin, and 10% fetal calf serum (Flow Laboratories) at 37°C in a 5% CO₂ humidified atmosphere in air. M14 ADR cell line was selected culturing M14 cells in the presence of 40 μM DOX (Adriblastina, Pharmacia & Upjohn S.P.A., Milan, Italy). All procedures were performed with written institutional review board approval.

**Chemicals** *M. alternifolia* essential oil (Pharmaceutical Grade) was kindly supplied by Variati (Milan, Italy). (+)-Terpinen-4-ol and 1,8-cineole were purchased from Fluka (Buchs, Switzerland) and Sigma-Aldrich (St Louis, Missouri), respectively. The oligopeptide caspase inhibitors z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), z-Phe-Ala-fluoromethylketone (z-FA-fmk), and z-Asp(Ome)-val-Asp-(Ome)-fluoromethylketone were purchased from Enzyme Systems Products (San Diego, California). CsA was obtained from Sigma (St Louis, Missouri).

**Gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS)** Gas chromatography equipment used included a Perkin Elmer AutoSystem (Perkin Elmer Corporation, Norwalk, CT) equipped with two fused-silica SPB 5 columns (60 m × 0.25 mm i.d., film thickness 0.25 μm), mounted in parallel in the same oven, with two detectors: FID and TurboMass Spectrometer (electron ionization 70 eV electron energy, transfer line 220°C). Carrier gas was oxygen and moisture-free helium obtained from SUPELCO TM High Capacity Heated Carrier Gas Purifier, provided with OMI-2 indicating tube, at the average flow rate of 1 mL per min. Oven temperature program was 60°C for 4 min, then 2°C per min to 180°C, then 3°C per min to 250°C. Detector
temperature was 280°C; the injector temperature was 280°C. The volume of the injected essential oil or reference substances ((-)-Terpinen-4-ol and 1,8-cineole) was 0.1 µL and the split ratio was 1:50. Two distinct data systems were connected to the GC-FID or GC-MS: Turbochrom and TurboMass Analytical Workstation Software with NIST/EPA/MSDC Mass Spectral database, respectively.

**Growth curves** M14 WT and M14 ADR cells (5 × 10⁶) were seeded in 24 multwell tissue culture plates. After 24 h, cultures were treated with TTO at concentrations ranging from 0.005 to 0.03% (v/v). Cells were then counted every day in a Neubauer chamber. Dead cells were subtracted by employing the Trypan blue exclusion method. The cell pool size represented the mean value of the number of cells collected from three wells.

**Cytotoxicity studies** The clonogenic survival test was used to determine the cell sensitivity to DOX. After treatment with DOX at the concentrations ranging from 0.42 to 60 µM for 1 h, cells were detached, plated (1000 per 60 mm tissue culture dish), and allowed to grow for 8 d. After growth, cell colonies were fixed with 95% ethanol, for 1 min, and stained with a solution of methylene blue in 80% ethanol, for 1 h. Only colonies composed of more than 50 cells were evaluated. The surviving fraction (SF) was calculated by dividing the absolute survival of cells treated with the drug (S_DOX) by the absolute survival of cells grown in drug-free medium (S_SFM) (SF = S_DOX/S_SFM × 10⁰). The values calculated depict the mean of three separate experiments. The resistance index was calculated as the ratio of the IC50 (inhibitory concentration of the growth of 50% cell population) of M14 ADR cells to the IC50 of M14 WT cells.

**Western blotting** Cultured cells were washed three times in cold phosphate buffer solution, and incubated for 30 min on ice in lysis buffer (150 µM NaCl, 50 µM HEPES, 10 µM EDTA, 1% Triton X-100) supplemented with protease inhibitor cocktail (one tablet per 10 mL of incubation solution) (Complete; Boehringer Mannheim GmbH, Mannheim, Germany). Debris and nuclei were pelleted by centrifugation at 10,000 g for 5 min. The protein concentration in the resulting supernatants was measured by using a DC Protein Assay (Bio-Rad Laboratories, Hercules, California). Samples of 30 µg of total protein were boiled in sample buffer and analyzed on 4% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred on to nitrocellulose membrane (Millipore Corporation, Bedford, Massachusetts). The membrane was probed with the primary monoclonal antibodies (MoAb) to P-gp, MoAb C219 (Signet Laboratories Inc., Dedham, Massachusetts) (work dilution 1:20) and to actin, MoAb 1501 (work dilution 1:5000) (Chemicon International Inc. Temecula, CA). All the incubations and washes were performed in PBS, containing 0.1% Tween 20. As secondary antibody, sheep anti-mouse immunoglobulin horseradish peroxidase-linked (whole antibody) (Amersham Pharmacia Biotech UK Limited, Little Chalfont, UK) was used, according to the manufacturer’s instructions. Detection was accomplished using the Enhanced Chemiluminescence detection kit (Amersham Pharmacia Biotech UK Limited). Experiments were repeated three times.

**Flow cytometry** For flow cytometric analysis, cell surface P-gp was labeled with MoAb MM4.17 (Cianfriglia et al. 1994). MoAb MM4.17 is an IgG₂κ monoclonal immunoglobulin reacting with a continuous linear epitope on the apex of the fourth loop of P-gp. This MoAb specifically recognizes a distinct human-specific epitope of the extracellular domain of the MDR1-P-gp isoform. The optimal concentration for these studies was 10 µg per mL.

For the determination of cell surface P-gp, the cells were incubated for 30 min at 4°C with MoAb MM4.17 in PBS, pH 7.2 containing 1% bovine serum albumin (Sigma), 10% fetal calf serum, and 10% human AB serum. After washing with ice-cold PBS cells were incubated for 30 min at 4°C with a F(ab′)₂ fragment of goat anti-mouse IgG-fluorescein conjugate (Sigma). After washing, cells were immediately analyzed. Dead cells were excluded from the analysis by adding propidium iodide (PI) to the cell suspensions before the acquisitions. For negative controls, cells were incubated with mouse IgG2a isotypic globulins.

For the measurement of annexin V binding, cells from exponentially growing control and TTO-treated cultures were collected at the time indicated, by adding the floating cells to the EDTA-detached ones and analyzing them together. Aliquots of 0.5 × 10⁶ cells were centrifuged (100 g) for 5 min and washed with incubation buffer (10 µM HEPES/NaOH, 140 µM NaCl, 5 µM CaCl₂). The cell pellet was resuspended in 200 µL of labeling solution containing 1 µL annexin V-fluorescein isothiocyanate labeling reagent (MBL, Medical & Biological Laboratory Co., Ltd, Naka-ku Nagoya, Japan) and incubated for 10 to 15 min. After adding 200 µL of incubation buffer, cells were washed and resuspended in 200 µL containing 1 µL PI solution. The apoptotic index (AI) was expressed as the percentage of cells binding annexin V but negative for PI. The in situ DNA fragmentation labeling was performed by the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling) method. Aliquots of about 10⁴ cells were fixed in formaldehyde (1% in PBS) for 15 min at room temperature. After, cells were rinsed in ice-cold PBS, incubated with ethanol for 2 min on ice, rinsed in ice-cold PBS and resuspended in TUNEL reaction mixture (Roche Molecular Biochemicals, Mannheim, Germany) for 60 min at 37°C in humidified atmosphere in the dark. After washing in ice-cold PBS, cells were analyzed by flow cytometry. In order to evaluate the viability of M14 WT and M14 ADR cells after treatment with TTO or terpinen-4-ol, in the presence or absence of caspase inhibitors, the trypan blue (Gibco BRL Life Technologies, Gaithersburg, Maryland) exclusion method was carried out. After treatment, cells were resuspended in ice-cold PBS, stained with trypan blue at a final concentration of 80 µM and immediately analyzed by flow cytometry. All fluorospheres were analyzed with a FACSscan flow cytometer (Becton Dickinson, Mountain View, California) equipped with a 15 mW, 488 nm, air-cooled argon ion laser. The fluorescence emissions were collected through a 530 nm band-pass filter for fluorescein and annexin V, a 575 nm band-pass filter for PI, a 670 nm band-pass filter for trypan blue, and acquired in log mode. At least 10,000 events were analyzed. The analyses were performed by CellQuest software (Becton Dickinson).

**Electron microscopy** For scanning electron microscopy (SEM) analysis, cells were grown on 12 mm glass coverslips and treated as above reported. At the indicated times, cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at room temperature for 30 min, postfixed with 1% OsO₄ in the same buffer, dehydrated through a graded ethanol series, critical point dried with CO₂, and gold coated by sputtering. Samples were examined with a Cambridge Stereoscan 360 scanning electron microscope (Cambridge Instruments Ltd, Cambridge, UK).

For analyses on freeze-fracture (FF) replicates, cells were fixed with 2.5% glutaraldehyde in the culture medium. After 20 min of fixation, cells were centrifuged for 10 min at 100 g, washed twice in Hank’s balanced salt solution, resuspended in the same medium containing 25% glycerol, 1% NaN₃, and incubated for 2 h at 4°C. The cell suspension was then centrifuged at 450 g for 15 min and the pellet was put on carriers and quickly frozen in Freon 22, partially solidified at the liquid nitrogen temperature. The mounted carriers were then transferred into a Bal-Tec BAF 060 freeze-etch unit (BAL-TEC Inc., Balzers, Liechtenstein), cleaved at a pressure of 2 to 4 × 10⁻⁷ mbar, shadowed with 2.5 nm of platinum-carbon and replicated with 20 nm carbon film. Platinum-carbon evaporation (at an angle of 45°) an carbon evaporation (at an angle of 90°) were performed using electron beam guns; the thickness of the deposit was evaluated by means of a quartz crystal thin film monitor. Cells were digested for 2 h from the replica by chlorox. The replicas were mounted on naked 300 mesh grids and examined with a Philips EM 208S electron microscope (FEI Company, Eindhoven, the Netherlands) at 60 kV.
Reverse transcription–polymerase chain reaction (reverse transcription–PCR) MDR1 mRNA was analyzed by the reverse transcription–PCR technique. Total cellular RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform extraction method using TRIZOL reagent (Gibco-BRL); the concentration of RNA was determined spectrophotometrically at 260 nm. RNA preparations were treated with Dnase I-Rnase-free (1 U per 5 μL, Boehringer-Mannheim, Milan, Italy) before cDNA synthesis. First-strand cDNA and amplification of specific DNA sequence were performed according to the manufacturer’s instructions (GeneAmp, RNA PCR Kit, Perkin Elmer Cetus, Emeryville, California). Briefly 1 μg of total RNA was used for cDNA synthesis using oligo(dt) priming in the presence of Moloney murine leukemia virus reverse transcriptase. The total cDNA reaction mixture (20 μL) was used for amplification of the MDR1. After an initial denaturation step at 94°C for 3 min, the cycling profile was: 94°C, 45 s denaturation; 54°C, 45 s annealing; 72°C, 1 min extension and quick chill to 4°C in a 9600 thermocycler (Perkin Elmer). A total of 40 cycles were performed. MDR1 specific sequences were amplified using the sense-strand primer CCTTCAACTTCTGCTCCTGA (residues 2733–2752), which yield a 167 bp product. The PCR products were fractioned in a 2% agarose gel and visualized by ethidium bromide staining. As a positive control, MCF7-DX cells expressing a high level of P-gp were employed. Several negative control reactions were included in each experiment. Experiments were repeated three times.

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EFFECTS OF TEA TREE OIL ON MELANOMA CELLS 359