Toxicology in Vitro 27 (2013) 323–329

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Toxicity of terpenes on fibroblast cells compared to their hemolytic potential and increase in erythrocyte membrane fluidity

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ARTICLE INFO

Article history:
Received 4 May 2012
Accepted 16 August 2012
Available online 27 August 2012

Keywords:
EPR
Spin label
Toxicity
Fibroblast
Terpenes
Erythrocyte membrane

ABSTRACT

Terpenes are considered potent skin permeation enhancers with low toxicity. Electron paramagnetic resonance (EPR) spectroscopy of the spin label 5-doxyl stearic acid (5-DSA) was used to monitor the effect of sesquiterpene nerolidol and various monoterpenes on membrane fluidity in erythrocyte and fibroblast cells. In addition, the hemolytic levels and cytotoxic effects on cultured fibroblast cells were also measured to investigate possible relationships between the cellular irritation potentials of terpenes and the ability to modify membrane fluidity. All terpenes increased cell membrane fluidity with no significant differences between the monoterpenes, but the effect of sesquiterpene was significantly greater than that of the monoterpenes. The IC_{50} values for the terpenes in the cytotoxicity assay indicated that 1,8-cineole showed lower cytotoxicity and α-terpineol and nerolidol showed higher cytotoxicity. The correlation between the hemolytic effect and the IC_{50} values for fibroblast viability was low ($R = 0.61$); however, in both tests, nerolidol was among the most aggressive of terpenes and 1,8-cineole was among the least aggressive. Obtaining information concerning the toxicity and potency of terpenes could aid in the design of topical formulations optimized to facilitate drug absorption for the treatment of many skin diseases.

1. Introduction

Terpenes are volatile constituents of the essential oils of citrus fruits, cherries, mints and herbs that contain only carbon, hydrogen and oxygen atoms. They can be chemically classified as alcohols, hydrocarbons, ketones and epoxides. Physiologically, terpenes function primarily as chemoattractants or chemorepellents (McGarvey and Croteau, 1995) and are largely responsible for the characteristic fragrance of many plants (Crowell, 1999). Recently, the potential of terpenes as potent skin permeation enhancers for drug delivery systems has received considerable attention, especially because they are naturally occurring substances with low skin irritation. Many terpenes, including 1,8-cineole, menthol and α-terpineol, are included on the list of "Generally Recognized As Safe" (GRAS) materials. Several monoterpenes show no change or only a slight irritation and cytotoxic effect on cultured human skin cells (Kitahara et al., 1993). In this context, skin permeation enhancers, particularly oxygen-containing terpenes, were used as accelerants of permeation for lipophilic drugs, such as 5-fluorouracil (Cornwell and Barry, 1994), morphine (Morimoto et al., 2002), imipramine (Jain et al., 2002), hydrocortisone (El-Kattan et al., 2000) and haloperidol (Vaddi et al., 2002).

A number of dietary monoterpenes have demonstrated antitumor activity and are effective in the chemoprevention and chemotherapy of cancer (Crowell, 1999; Rabi and Bishayee, 2009; Thoppil and Bishayee, 2011; Gould, 1997; Bardon et al., 1998, 2002; Wu et al., 2012; Yang and Ping Dou, 2010; Polo and de Bravo, 2006). The monoterpenes linalool, carvacrol, geraniol and terpinen-4-ol have shown activity against Leishmania *infantum* promastigotes (Morales et al., 2009). Moreover, terpinen-4-olo and the sesquiterpene nerolidol were reported to show antifungal (Oliva et al., 2003) and antileishmanial activity (Arruda et al., 2005), respectively.

Electron paramagnetic resonance (EPR) spectroscopy of spin labels has been recently used to investigate the mechanisms underlying the action of terpenes as accelerants of skin permeation. The intercellular membranes of the stratum corneum, which is the outermost skin layer and primary physical barrier for skin permeation, become fluid in presence of the terpenes L-menthol (Dos Anjos et al., 2007) and 1,8-cineole (Anjos et al., 2007). In addition, treatment with monoterpenes increases the partition coefficient of the small water-soluble spin labels TEMPO (Dos Anjos and Alonso, 2008) and DTBN (Camargos et al., 2010) into stratum corneum membranes. These results suggest that terpenes might effectively act as spacers in the membrane to fluidize lipids and create

Abbreviations: EPR, electron paramagnetic resonance; 5-DSA, 5-doxyl stearic acid.

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http://dx.doi.org/10.1016/j.tiv.2012.08.022

ruptures in the hydrogen-bond network of the polar interface (Dos Anjos and Alonso, 2008).

Few studies have investigated whether the ability of terpenes to facilitate chemical absorption correlates with increased irritation potentials. Terpenes are important skin permeation enhancers for drug delivery systems; therefore, we investigated the effect of nerolidol, α-terpineol, L(−)-carvone, (+)-limonene, L-menthone, DL-menthol, pulegone and 1,8-cineole on erythrocyte membrane fluidity. Moreover, the hemolytic potentials and toxicity levels of these terpenes on fibroblast cells were also investigated.

2. Materials and methods

2.1. Chemicals and preparation

Materials for the 3T3 Neutral Red Uptake (NRU) assay and the spin label 5-doxyl stearic acid (5-DSA) (Fig. 1) were purchased from Sigma–Aldrich (St. Louis, MO, USA; Steinheim, Germany). The terpenes were purchased from Acros Organics (Geel, Belgium). All other chemicals were of the highest grade available and the buffers were prepared with Milli-Q water.

2.2. Cell culture

Balb/c 3T3-A31 fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM – D5648 Sigma–Aldrich), supplemented with 10% fetal bovine serum (FBS-GIBCO) at 37 ± 1 °C, 90 ± 10% humidity, 5.0 ± 1.0% CO_2/air. The cells were removed from the culture flasks using trypsinization (trypsin:EDTA solution at a 0.25%:0.02% ratio) when they exceeded 50% confluence but prior to reaching 80% confluence. Cell viability was evaluated using the Trypan blue exclusion method with a Neubauer chamber.

A cell suspension containing 3×10^4 cells/mL was prepared on the day of plate seeding using culture medium supplemented with 10% FBS. The peripheral wells (blanks) of the 96-well microtiter plates were seeded with 100 μL of routine culture medium and the remaining wells received 100 μL of a suspension containing 3×10^4 cells/mL (3×10^3 cells/well). The plates were incubated for 24 ± 2 h (37 ± 1 °C; 90 ± 10% humidity, 5.0 ± 1.0% CO_2/air) to allow the cells to form a monolayer of less than 50% confluence. This incubation period assured cell recovery, adherence and progression to the exponential growth phase. Each plate was examined under a phase contrast microscope to identify experimental and systemic cell seeding errors.

2.3. The Neutral Red Uptake (NRU) assay

For *in vitro* assays, the terpenes (nerolidol, α-terpineol, L(−)-carvone, (+)-limonene, L-menthone, DL-menthol, pulegone or 1,8-cineole) were prepared individually as a micellar suspension to allow dissolution in water. The micelles were prepared as follows: 10 mg of phosphatidylcholine (PC) and 50 μL of the terpenes to be tested were dissolved in 50 μL of ethanol. The mixture was sonicated for 10 min in a Ti-probe sonicator to obtain a homogeneous dispersion of small micelles. The micellar suspension was prepared without terpenes for control groups. The experimental samples were directly diluted in culture medium (DMEM) to obtain the concentration of use and filtered through a syringe-filter with a PES TPP® membrane (0.22 μm pore size) to assure sterility. The final concentration of ethanol in all cultures was lower than 0.05%.

A Balb/c 3T3-A31 cell suspension containing 3×10^4 cells/well was seeded in 96-well plates, and after a 24 h recovery period, the plates were treated with eight different concentrations of freshly prepared test compounds in complete medium (six wells per concentration) and incubated for an additional 48 h. The control wells (blanks) received complete culture medium supplemented with 10% FBS. Subsequently, 250 μL of neutral red (NR) medium was added to all wells, including the blanks, and incubated (37 ± 1 °C, 90 ± 10% humidity, 5.0 ± 1.0% CO_2/air) for 3.0 ± 0.1 h. The cells were briefly observed 2–3 h after incubation for NR crystal formation. After 3 h, the NR medium was removed and the cells were carefully rinsed with 250 μL/well of pre-warmed PBS. The PBS was decanted from the plate and 100 μL of NR desorb (50:1:49 EtOH:acetic acid:water) solution was added to all wells, including the blanks. The plates were rapidly shaken on a microplate shaker for 20 min to extract the NR. The absorption was measured at 545 nm in a microtiter plate reader (spectrophotometer). The optical density (OD) was calculated as the difference between the absorbances at the test wavelength and that at the reference wavelength. For each concentration tested, the wells containing no cells served as reference blanks.

2.4. Blood processing

The blood samples, obtained from three donors of two blood banks, were diluted in PBS and centrifuged at 150*g* for 10 min at 4 °C. The plasma and white cells were carefully removed after each wash (three times).

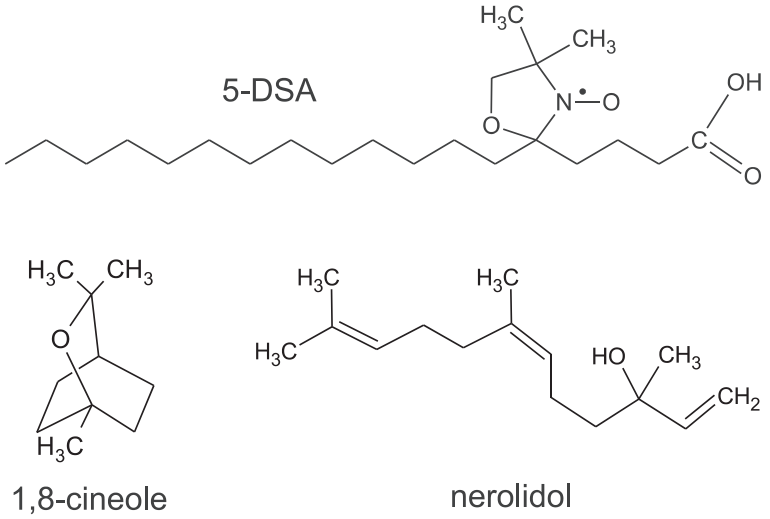

Fig. 1. The chemical structures of two terpenes and the spin label 5-DSA used in this work.