Abnormal mitotic spindle assembly and cytokinesis induced by D-Limonene in cultured mammalian cells

Maurizio Mauro, Irene Catanzaro, Flores Naselli, Giulia Sciandrello and Fabio Caradonna*

Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF, sezione di Biologia Cellulare) Università di Palermo, Viale delle Scienze, Edificio 16, 90128 Palermo, Italy.

*To whom correspondence should be addressed. Tel: +39 9123897331; Fax: +39 916577347; Email: fabio.caradonna@unipa.it

D-Limonene is found widely in citrus and many other plant species; it is a major constituent of many essential oils and is used as a solvent for commercial purposes. With the discovery of its chemotherapeutic properties against cancer, it is important to investigate the biological effects of the exposure to D-Limonene and elucidate its, as yet unknown, mechanism of action. We reported here that D-Limonene is toxic in V79 Chinese hamster cells in a dose-dependent manner. Moreover, to determine the cellular target of D-Limonene, we performed morphological observations and immunocytochemical analysis and we showed that this drug has a direct effect on dividing cells preventing assembly of mitotic spindle microtubules. This affects both chromosome segregation and cytokinesis, resulting in aneuploidy that in turn can lead to cell death or genomic instability.

Introduction

D-Limonene is a monoterpene found especially in citrus essential oils. As well as its commercial use as an ingredient in many flavouring products, it is now an environmentally acceptable organic solvent for industrial purposes (1). Following oral administration, D-Limonene is absorbed rapidly and metabolised to perillic acid, dihydroperillic acid, limonene1,2-diol and uroterpenol (2). D-Limonene and its metabolites are not genotoxic, when tested in *Salmonella typhimurium* in the presence or absence of S9 fraction from rat liver (3). However, it has been found to be toxic *per se* in human lung cells under oxygen conditions similar to '*in vivo*' (1), and it showed genotoxicity at doses between 97 and 1549 mM when it is contained in essential oil of flowering plants (3).

D-Limonene has been reported to be tumourigenic in the kidney of male rats, although not in female rats or in other species. This can be explained by the interaction of D-Limonene and $\alpha_{2\mu}$-globulin, a low-molecular-weight protein found in significant quantities only in male rats (4). Thus, D-Limonene has been regarded as an animal carcinogen that is not expected to be carcinogenic to humans (5), leading the International Agency for Research on Cancer to classify it in the group 3 (not carcinogenic to humans). Interestingly, other studies have shown that D-Limonene possesses significant chemotherapeutic and chemopreventive properties against a number of murine cancers during the initiation phase, probably due to the monoterpene ability to induce carcinogen-metabolising enzymes (6). More recently, it has been reported that D-Limonene exhibits chemopreventive efficacy in preclinical hepatocellular carcinoma models (7) and essential oils from *Lippia alba* against bleomycin-induced genotoxicity.

In a previous work, we have evaluated the toxicity of several terpenes present in a citrus essential oil (α-Pinene, β-Pinene, D-Limonene and 3-Carene) that were found in high concentrations in actual industrial effluents. D-Limonene resulted not mutagenic in *S. typhimurium* or in V79 Hamster Chinese cells (8), but it is capable to induce DNA damage. This ability, evaluated by comet assay, may be the result of a direct genotoxicity or an indirect effect, i.e. the terpenes capability of inducing reactive oxygen species (ROS) (9). Despite the fact that D-Limonene is widely found in the environment and exposure both indoors and in the workplace is common, its mechanism of action is poorly understood.

In this study, *in vitro* experiments were conducted to better explore the target(s) of D-Limonene. We demonstrate that acute D-Limonene exposure prevents assembly of mitotic spindle microtubules that in turn can affect both chromosome segregation and cytokinesis leading to cell death or genomic instability.

Materials and Methods

Cell culture

The V79 Chinese hamster cells were routinely cultured in Dulbecco's modified Eagle's medium (Biochrome) supplemented with 5% fetal bovine serum (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (25 μg/μl and maintained at 37°C in a 5% CO_2 humidified incubator as previously reported (10). These cells have a doubling time of 14–16 h.

D-Limonene treatment

D-Limonene (Carlo Erba, CAS: 5989-27-5) was further purified by distillation; its purity was verified by gas chromatography in a mass spectrometer detector. For cell treatments, D-Limonene was diluted in dimethyl sulfoxide (DMSO; Sigma–Aldrich, CAS: 67-68-5); the final concentration of DMSO was < 0.5%.

Clonogenic assay

Cells (300) were plated in six-well plates and 18 h after seeding were exposed to increasing doses of D-Limonene (0, 0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 mM) for
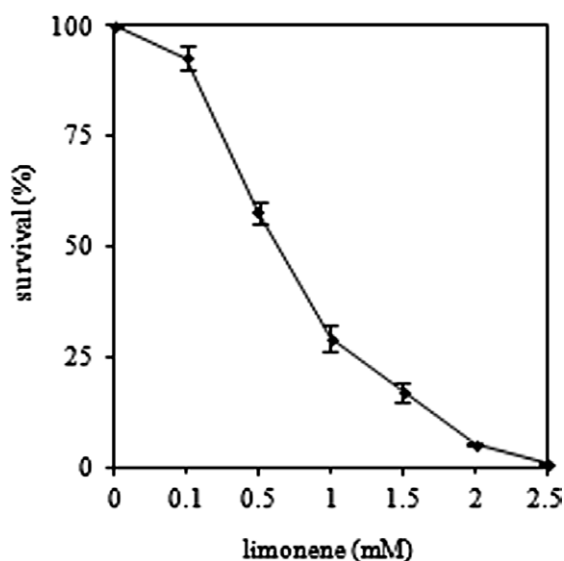

Fig. 1. Cell survival in increasing concentrations of D-Limonene. Data are presented as the mean ± SD percentages of three independent experiments.

1 h in complete medium. After treatment, the cells were washed twice with Hank's salt solution and incubated in fresh medium for 10 days. The colonies were then stained with 0.1% methylene blue. Treatments were performed three times, and cell survival was expressed as a percentage of untreated cells as in the previously reported procedure (11).

Morphological observation

Cells (5.0×10^5), plated on 18×18 mm glass coverslips in 60-mm Petri dishes, were exposed for 1 h in complete medium to 0, 0.5, 1.0 and 1.5 mM D-Limonene 18 h after seeding. Cells were then washed twice with phosphate-buffered saline (PBS) and incubated in fresh medium for 0, 3, 6, 9, 12 and 24 h. For morphological analyses, cells were prefixed with methanol added to the culture medium at a ratio of 1:1 for 5 min at room temperature, fixed with methanol for 7 min and then stained with 2.5% Giemsa. At least 1000 cells (minimum 1000 to maximum 1200) were examined each time to determine abnormal cell frequencies. Three independent experiments were performed.
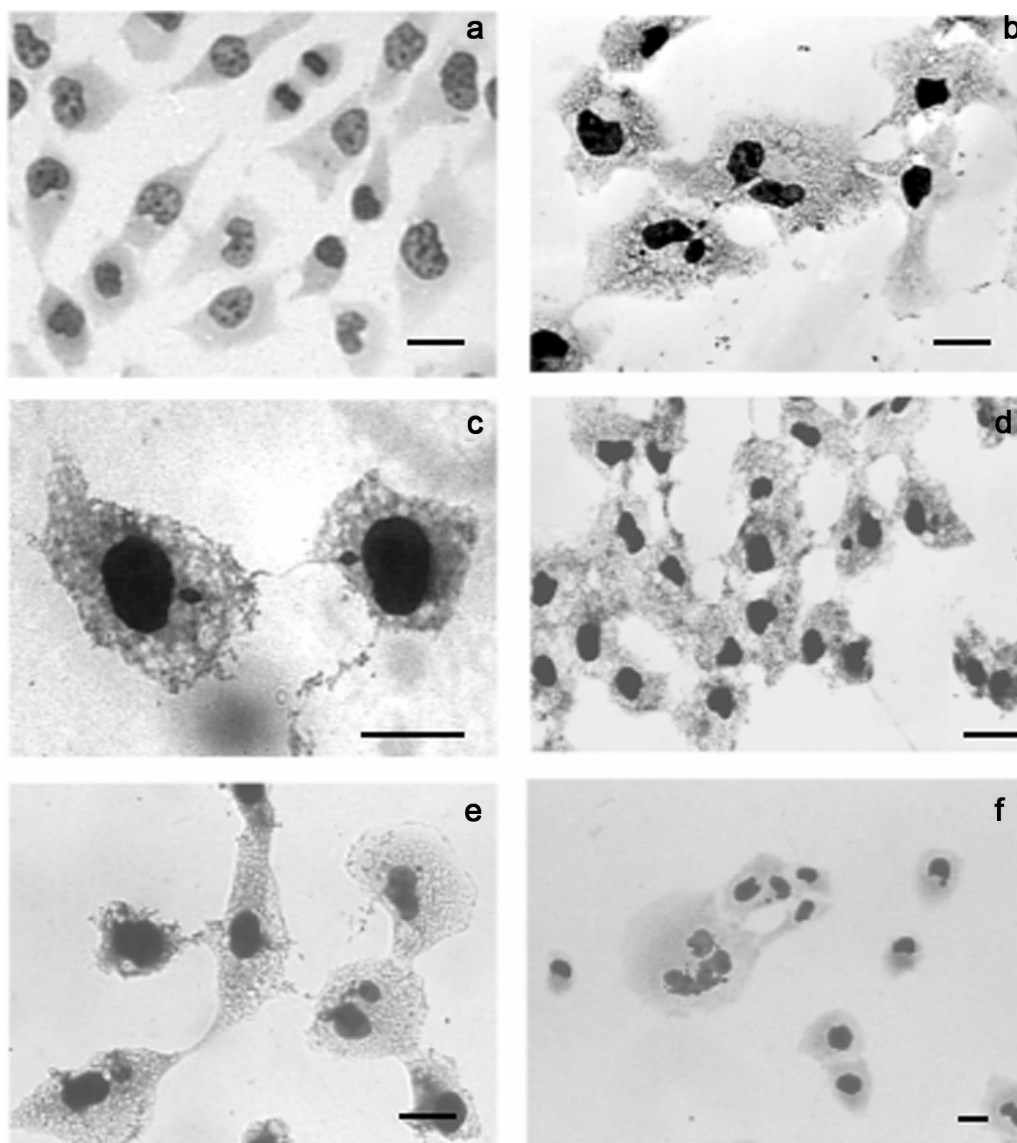
Micronuclei assay using anti-kinetochore antibody

The method used was essentially that described by Degrassi and Tanzarella (12) with some modifications. Briefly, 5.0×10^5 cells were plated on 18×18 glass coverslips in 60-mm Petr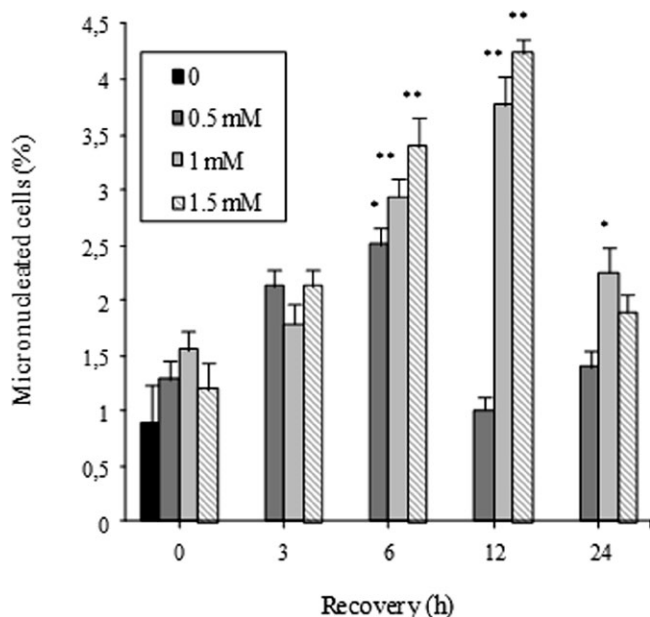i dishes and exposed to 1.5 mM D-Limonene for 1 h, then examined after post-treatment incubation in fresh medium for 0 and 6 h. The slides were immediately fixed in absolute methanol precooled to $-20°C$ for 30 min. After rinsing four times in PBS + 1% bovine serum albumin (BSA) with 0.1% Triton X-100, the samples were incubated overnight at 4°C with anti-kinetochore antibody (Antibodies Incorporated, Davies, CA, USA). The samples were washed five times with the same washing solution and then incubated for 60 min at 37°C with fluorescein isothiocyanate-conjugated goat anti-human IgGs (Sigma) diluted 1:100 in PBS–BSA. After washing, the preparations were mounted in antifade solution containing 2 µg/ml propidium iodide as a counterstaining agent. The slides were observed under a Nikon fluorescence photomicroscope equipped with a HBO 100W mercury lamp and a suitable filter at ×100 magnification. Between 100 and 200 micronuclei per experimental point were evaluated.
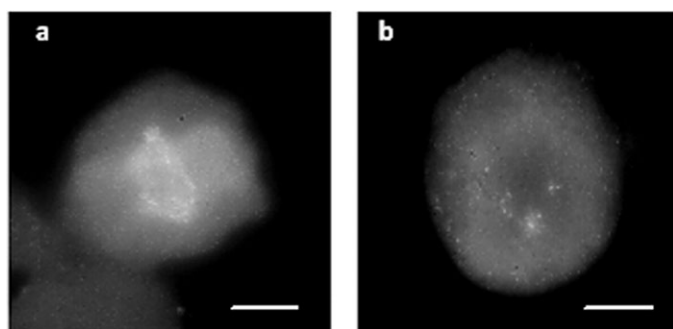
Indirect immunostaining

For the microtubule analysis, cells plated onto glass coverslips, as reported above, were exposed for 1 h to 0 and 1.5 mM D-Limonene. Cytochalasin B (Sigma), at the most appropriate concentration and treatment time, was used as positive control for 16 h to 3 µg/ml. At the end of treatments, cells were washed, fixed and then incubated with anti-β tubulin antibody, as previously described (13).
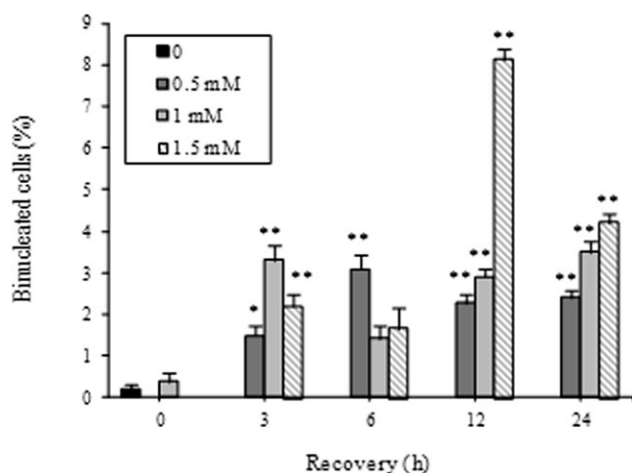

Fig. 2. D-Limonene induces nuclear abnormalities such as micronuclei (**b**), micronuclei with chromosome bridge (**c**), hyperchromatic nuclei (**d**), cytoplasm thickening (**e**) and giant multinucleated cell (**f**). Cells recovered after 3 h (**b**), 6 h (c and d) and 6 h (e and f) post-treatment with 1.5 mM D-Limonene; untreated cells (**a**). Bars represent 10 µm.

Fig. 3. Frequencies of micronucleated cells during 24 h recovery from treatment with increasing concentrations of D-Limonene. Data are presented as the mean ± SD percentages of three independent experiments. Statistically significant differences versus untreated cells are indicated as *$P < 0.01$ and **$P < 0.005$, according to the Student's t-test.

Fig. 4. Examples of normal (**a**) and D-Limonene-induced aberrant (**b**) mitotic spindles following indirect immunostaining with β-tubulin antibody. DNA was counterstained with propidium iodide. Bars represent 2 μm. ×100 magnification.

Fig. 5. Frequencies of binucleated cells during 24 h recovery from treatment with increasing concentrations of D-Limonene. Data are presented as the mean ± SD percentages of three independent experiments. Statistically significant differences versus untreated cells are indicated as *$P < 0.01$ and **$P < 0.005$, according to the Student's t-test.

Image processing

All photomicrographs for morphological observations and immunofluorescence analysis were processed using Adobe Photoshop 3.0 LE software.

Results and Discussion

D-Limonene is rapidly absorbed and metabolised to oxygenated metabolites in rats and humans (14). However, it has been recently demonstrated that the D-Limonene concentrates in the breast tissue, while the major active circulating metabolite, perillic acid, does not (15). In another study, it was found that D-Limonene accumulates in adipose tissue after oral dosing in humans (16). Several studies have focused on the effects of D-Limonene metabolites in human and mouse models prompting us to detect the cellular target(s) of D-Limonene rather than its metabolites. To reach this goal, we used purified D-Limonene and chose a cellular system that does not metabolise monoterpenes having no cytochrome P450 activity (17).

Dose–response experiments showed that increasing concentrations of D-Limonene had increasingly toxic effects on V79 Chinese hamster cells (Figure 1). Indeed, using clonogenic assay, we calculated an ID_{50} of 0.75 mM for D-Limonene in V79 cells. In particular, concentrations >1 mM resulted in extensive cell death.

To gain insight into D-Limonene cytotoxicity and its potential target(s), we chose to use the concentrations of 0.5, 1 and 1.5 mM, one below and two above the ID_{50}, respectively, for further experiments on V79 cells.

Morphological observations performed up to 24 h after treatment with increasing doses of D-Limonene showed cells with altered morphology and nuclear abnormalities compared with the untreated cells also analyzed for each recovery time independently of dose and recovery time. These changes were often associated with cytoplasm thickening (Figure 2).
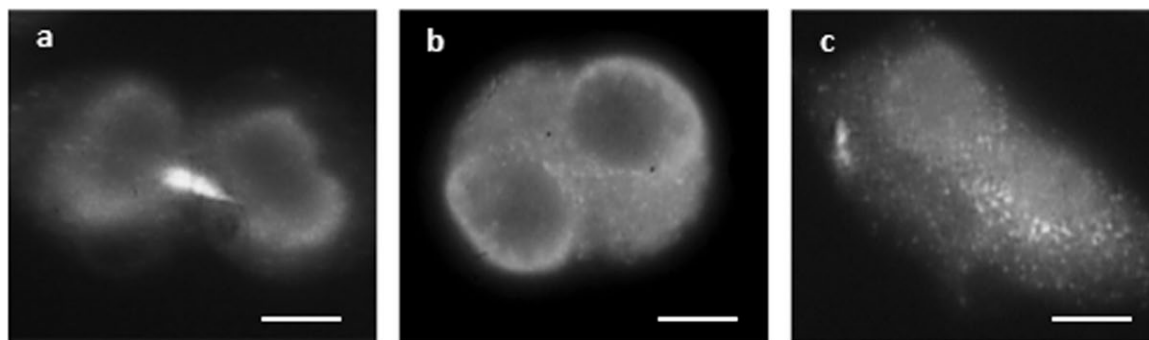
Among the cells with nuclear abnormalities, micronucleated cells were seen with significant frequency compared with untreated cells after a recovery of only 6 h following treatment with 0.5 mM D-Limonene. In contrast, this frequency decreased after 24 h recovery (Figure 3).

Taking into account that micronuclei originate from either acentric chromosome fragments or from whole chromosomes that are not incorporated into daughter nuclei at the time of cell division, our result strongly suggests that D-Limonene most likely targets cells entering in mitosis. We next sought to distinguish micronuclei caused by chromosome breakage from those due to chromosome loss, by using immunofluorescent staining of kinetochores. Indeed, micronuclei with acentric fragments, that are not expected to contain kinetochore proteins, are kinetochore negative, while those containing whole chromosomes are kinetochore positive.

Results from indirect immunofluorescence with anti-kinetochore antibody showed the ability of D-Limonene to induce chromosome loss as most micronuclei (70%) were kinetochore positive, compared with 4% of those found in untreated cells. As we also noticed a valuable presence of 30% of kinetochore-negative micronuclei D-Limonene induced (comparable to 96% of those found in untreated cells), we cannot exclude that acute D-Limonene exposure can also cause chromosome breakages most likely due to increase in ROS. However, further experiments are needed to clarify this finding.

It has been extensively demonstrated that lagging chromosomes can generally arise by a malfunction in the mitotic spindle apparatus, such as disruption of the microtubules. For instance, colchicine, a very well-known antimicrotubule agent,

Fig. 6. Examples of normal cytokinesis **(a)**, ᴅ-Limonene-induced **(b)** and Cytochalasin B-induced **(c)** cytokinesis failure following indirect immunostaining with β-tubulin antibody. DNA was counterstained with propidium iodide. Bars represent 2 µm. ×100 magnification.

is a potent kinetochore-positive micronuclei inducer (18). Consistently, indirect immunofluorescence with anti-β tubulin antibody showed that half of the treated cells had only faintly fluorescent spots inside metaphase plates; this probably represents a failed attempt to nucleate microtubule assembly raising the possibility that ᴅ-Limonene affects the proper building of the mitotic spindle (Figure 4).

Another interesting result of the morphological analysis was the occurrence of binucleated cells whose frequency was always significantly higher than in untreated cells. The highest occurrence of binucleated cells was observed after treatment with 1.5 mM ᴅ-Limonene and a recovery of 12 h (Figure 5). On the other hand, elevated level of multinucleated cells 24 h post-exposure were scored (data not shown), indicating that even binucleated cells underwent to aberrant mitosis. Interestingly, these data might suggest that ᴅ-Limonene excretion is not complete within 24 h post-exposure. However, further experiments are necessary to establish the amount of ᴅ-Limonene excreted over a certain period of time.

Binucleated cells arise from the lack of the contractile ring that determines the cytokinesis block. In fact, to obtain binucleated cells for research use, cells are exposed to Cytochalasin B. Cytochalasin B is an agent that disrupts microfilaments and prevents cytokinesis leading to the production of binucleated cells (19).

To further understand the effects of ᴅ-Limonene, cells were treated with either ᴅ-Limonene or Cytochalasin B and then incubated with anti-β tubulin antibody. As shown in Figure 6, the midbodies in binucleated cells induced by ᴅ-Limonene appeared to be broken up into scattered grains, whereas those in binucleated cells induced by Cytochalasin B were compact, although sometimes present at a distance from the centre. This finding, which demonstrates that microtubule bundle failure in the central spindle results in failed cytokinesis, provides evidence that ᴅ-Limonene gives rise to binucleated cells through a different mechanism from Cytochalasin B. It is worthwhile to mention that Cytochalasin B affects normal F-actin turnover producing aberrant furrows in late mitosis, while no detectable effect on stages of mitosis up to late anaphase was found (20).

Taken as a whole, the results reported here demonstrate that one cellular target of ᴅ-Limonene is the mitotic spindle microtubules most likely by causing depolymerisation of tubulin in the early stage of mitosis. The direct effect of this drug in preventing microtubule assembly affects both chromosome segregation and cytokinesis, resulting in aneuploidy that in turn can leads to cell death or induction

of unstable cells, with consequent long-term effects. However, further investigation will be necessary for a better understanding of ᴅ-Limonene mechanism of action. A first step would be to evaluate the destiny of bi-multinucleated cells over the time of our study and how ᴅ-Limonene affects microtubule metabolism. It would be also interesting to understand if ᴅ-Limonene interacts directly or indirectly with the DNA. More importantly, perhaps, would be to evaluate the dose response of cells/target tissues, especially using prolonged low-dose exposure.

Conflict of interest statement: None declared.

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