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Limonene Exerts Antiproliferative Effects and Increases Nitric Oxide Levels on a Lymphoma Cell Line by Dual Mechanism of the ERK Pathway: Relationship with Oxidative Stress

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ABSTRACT

D-limonene is a common monoterpene found in nature. Previously, it has been demonstrated that it has antiproliferative effects on a lymphoma cell line by increasing the nitric oxide (NO) level. In the present work this mechanism is analyzed by evaluating the participation of MAP38 and ERK pathways. Limonene increased the NO levels by inducing cell apoptosis by two mechanisms: through the production of H_2O_2 and ERK pathway activation at low concentrations, and through the inhibition of farnesylation of proteins and O_2^- production at high concentrations. Both mechanisms were related to the increase in total nitrites, and the production of oxidative stress intermediates was involved.

INTRODUCTION

D-limonene is one of the most commonly found monoterpene in nature. It is found in several citrus oils such as orange, lemon, mandarin, lime, and grapefruit. D-limonene is listed in the Code of Federal Regulations as generally recognized as safe (GRAS) flavoring agent and can be found in common food items such as fruit juices, soft drinks, baked goods, ice cream, and puddings. D-limonene has been shown to inhibit the development of spontaneous neoplasms in mice and to reduce the incidence of spontaneous lymphomas in $p53^{-/-}$ knockout mice (1). Moreover, it has been reported to inhibit the development and produce regression of rodent mammary cancer chemically induced by N-nitroso-N-methylurea (NMU) and 7, 12-dimethylbenz[a]anthracene (DMBA) (2, 3). A previous study has identified the presence of limonene in *Tilia x viridis* extract, as an active compound with an antiproliferative activity on a lymphoma cell line (BW5147) (4). Moreover, in these cells, nitric oxide (NO) was involved in the antiproliferative effect of limonene. The increase of NO levels was related to cell apoptosis and cell cycle arrest induced by different concentrations of limonene (5). Nevertheless, the mechanism by which limonene exerted the increase in NO levels was not studied.

The participation of the ERK pathway has also been demonstrated in the production of NO by different concentrations of exogenous hydrogen peroxide (H_2O_2) in BW5147 cells. Moreover, in the same cell line, the P-38 pathway was also involved in apoptosis produced by H_2O_2 through NO induction (6). Besides, the increase of reactive oxygen species (ROS) production has long been associated with the apoptotic response induced by several anticancer agents (7).

The status of intracellular redox potential is regulated by antioxidant enzymes (SOD, catalase, and glutathione peroxidase) together with other non-enzymatic antioxidants (8).

In this work, the molecular mechanism involved in the increase of NO levels and on the antiproliferative effect exerted by limonene in BW5147 cells was studied, analyzing the participation of ERK and P-38 pathway, which are involved in both NO production and the modulation of cell proliferation. The

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enzymes related to the generation and destruction of reactive oxygen species (ROS) were also evaluated.

MATERIALS AND METHODS

Cell suspensions and culture conditions

The BW5147 tumor cell line (Institute fur Virologie und Immunobiologie der Universitat Wurzburg, Germany) is a murine T-cell lymphoma that expresses the H-2 k haplotype, CD3, and $\alpha\beta$ T-cell receptor. These cells were cultured at optimal concentrations of 1–5 $\times$ 10^5 cells/mL in RPMI 1,640 medium (GIBCO, NY, USA) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, and antibiotics, and settled at a final volume of 0.2 mL in 96-well flat-bottom microtiter plates (Nunc) for microculture. Cells were cultured during different time periods; 24 hr was selected as the time of maximum response according to kinetic studies.

Proliferation assays and viability studies

The effect of limonene on cell proliferation was evaluated by the uptake of tritiated thymidine [3H]TdR. Cells were pulsed with 20 Ci/mmol [3H]TdR for 6 hr, as described previously (9). The effect of limonene was studied alone or in the presence of different inhibitors of the pathways involved in the control of cell proliferation: 5 $\times$ 10^{-6} M UO126 (Cell Signalling Technologies, Danvers, MA, USA), a MEK1/2 inhibitor; 1 $\times$ 10^{-4} M mevalonic acid (Sigma, San Diego, USA), an inhibitor of farnesylation of P21 Ras proteins; 10 $\times$ 10^{-6} M SB 203580 (Calbiochem, San Diego, USA), an inhibitor of P38; 10^{-3} M L-N-monomethylarginine (LNMMA) (Sigma, San Diego, USA), an inhibitor of NOS activity; 2 $\times$ 10^{-6} M Rotenone (Sigma, San Diego, USA), an inhibitor of transfer of electrons, and 6 mM sodium azide, a MnSOD inhibitor.

Results were expressed as percentage of inhibition: [1-dpm treated/dpm control] $\times$ 100. Data represent the media $\pm$ SEM of three experiments performed in triplicate. Cell viability was determined on tumoral lymphocytes incubated during 24 hr in the presence or absence of different concentrations of limonene, using two methods: trypan blue exclusion method and MTT. The viability was expressed as percentage of living cells (10).

Apoptosis assays

To determine whether limonene-induced nuclear changes are compatible with apoptosis, 10^5 tumoral lymphocytes were incubated in 24-well plates during 24 hr with and without different concentrations of limonene alone or in the presence of P-38 inhibitor, MEK1/2 inhibitor, and mevalonic acid. Cells were then washed twice with phosphate buffer solution (PBS), resuspended in 1 $\times$ binding buffer at a concentration of 1 $\times$ 10^6 cells. Aliquots of 1 $\times$ 10^5 cells/mL were incubated with annexin V-FITC (Sigma, San Diego, USA) and propidium iodide (Sigma, San Diego, USA) for 15 min, at room temperature, in the darkness. Samples were gently shaken, diluted with binding buffer, and analyzed by flow cytometry within 1 hr after incubation. An apoptosis control was done, incubating the cells in the same culture medium, but without FCS during 48 hr.

Total nitrite determination

The effect of limonene on total nitrites was determined using the Griess reagent (11). Cells were incubated with different concentrations of limonene alone or in the presence of L-NNMA, MEK1/2 inhibitor, respiration inhibitor, sodium azide, and mevalonic acid, at the same concentrations as used in the proliferation assays. Cells were incubated during 4 hr. A control sample of cells in 0.5% ethanol alone was added. After incubation, cell culture supernatants were collected and centrifuged at 800 $\times$ g for 10 min, then incubated with Griess reagent for 20 min in darkness, and finally the absorbance was measured at 540 nm. Total nitrites were calculated by interpolation in a standard curve made with the known concentrations of nitrites.

iNOS western blot

To analyze the iNOS induction, cells were incubated with limonene at 40 μg/mL with/without MEK1/2 inhibitor during 4 hr and limonene 100 μg/mL for 24 hr. For the preparation of whole cell lysate samples, treated and untreated tumor cells were centrifuged for 10 min, at 800 $\times$ g, and cell pellets were dissolved in sample buffer (2% SDS, 10% (vol/vol) glycerol, 62.5 mM Tris· HCl, pH 6.8, 0.2% bromphenol blue, and 10 mM 2-mercaptoethanol) at a final concentration of 10 mg/mL. Equal amounts of proteins were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes (12). Nonspecific binding sites in nitrocellulose membranes were blocked with blocking buffer (5% non-fat dry milk containing 0.1% Tween 20 in 100 mM Tris· HCl, pH 7.5, and 0.9% NaCl) overnight. The nitrocellulose membrane was subsequently incubated with a protein G-conjugated anti-iNOS isoenzymes for 18 hr. As control for protein loading, a Protein G-anti-β_2-microglobulin (Santa Cruz Biotechnology, Santa Cruz, USA) was used. The membrane was then incubated with a secondary monoclonal antibody conjugated to alkaline phosphatase (Sigma, San Diego, USA) for 1 hr. Immunoreactive bands were visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. A full range rainbow molecular weight markers were used. The same samples were run in 15% SDS-polyacrylamide gels and developed with an anti-β_2-microglobulin antibody (Santa Cruz Biotechnology) as an internal control for protein loading.

NF-kB ASSAYS

The effect of limonene (40 and 100 μg/mL) on NF-κ B translocation, on tumoral cells was determined by incubating the cells for 2 hr in the absence or presence of MEK1/2 inhibitor (5 $\times$ 10^{-6} M), by employing the Cayman Chemical NF-κ B (p65) (Cat. 10007889) transcription factor assay.

SUPEROXIDE AND PEROXIDASE ACTIVITY ASSAYS

Cells suspensions

Tumor cells (3×10^5 cells/mL) were incubated for 1 and 24 hr with limonene at 40 and 100 μg/mL in RPMI. After the addition of 10 μL of 10^{-4} M PMSF and 5 μL of Triton $\times$ 100, these cells were disrupted by pippeting and then centrifuged at 1,500 $\times$ g, for 15 min at 4°C. After centrifugation, the supernatant was used for the determination of enzymatic activity.

Oxygen-reducing activity: Superoxide dismutase (SOD) activity

About 50 μL of each cell supernatant was diluted with 910 μL of sodium phosphate buffer (0.05 M, pH 10.7) and 40 μL of adrenaline (final concentration 2 mM) was added immediately before the beginning of the OD monitoring at 480 nm kinetic mode. Results were expressed as the units (U) of SOD activity/mL, where 1 U of SOD induces the inhibition of auto-oxidation of adrenaline by 50% (13).

Hydrogen peroxide scavenging activity: Peroxidase activity

Supernatants were incubated in Krebs–Henseleit buffer (pH 7.4) containing: 125 mM NaCl, 4.0 mM KCl, 0.5 mM NaH_2PO_4, 0.1 mM $MgCl_2$, 1.1 mM $CaCl_2$, and 5.0 mM glucose; bubbled with 95% O_2 and 5% CO_2 at 37°C. The peroxidase activity was determined by the method described by Herzog and Fahimi (14). Briefly, 25 μL of each sample was incubated with 950 μL of 5×10^{-4} M 3′, 5′ diaminobencidine tetrahidrochloride (DAB) and 25 μL of H_2O_2 (a solution of 30% H_2O_2, parafarm R, diluted to 1/86 in distilled water) in a final volume of 1 mL. A DAB solution without H_2O_2 was used as reaction blank. In all cases, the reaction was initiated by the addition of H_2O_2 and the change in OD readings was recorded at 30 s intervals for 5 min using a Shimadzu recording spectrophotometer UV-240 (graphic printer PR-1) set at 465 nm. The Δ absorbance/min was calculated. A calibration curve of peroxidase concentration vs. Δ absorbance/min was plotted using horseradish peroxidase for obtaining a linear relationship in the range of 1.95×10^{-3}–2.5×10^{-5} U/mL. The activity of samples was calculated by the interpolation in the standard curve.

Release of H_2O_2

The determination of H_2O_2 was carried out by incubating tumoral (3×10^5 cells/mL) with limonene at 40 and 100 μg/mL alone or in presence of the MEK1/2 inhibitor in a solution of 0.56 mM of DAB containing 140 mM NaCl, 10 mM potassium phosphate, 5.5 mM dextrose, and type-II horseradish peroxidase 0.01 mg/mL (Sigma, San Diego, USA). After 1 hr incubation, the reaction was stopped by the addition of 10 mL of 4 N NaOH, and the OD was measured at 650 nm in a microplate reader (Microplate Reader Benchmark. Bio-Rad, CA, USA). Results were expressed as nanomoles of $H_2O_2/10^6$ cells. A standard curve of the known molar concentrations of H_2O_2 in buffered phenol red was run in each test.

Production of superoxide anion

Tumoral cells (3×10^5 cells/mL) were incubated with 100 μg/mL of limonene alone or limonene 100 μg/mL in the presence of mevalonic acid during 24 hr. Then the cells were centrifuged for 10 min at 800 $\times$ g, the supernatant was discarded, and cells were incubated with 300 μL of nitroblue tetrazolium (NBT) for 30 min in phosphate buffer. The reaction was stopped with 1 N HCl. Formazan produced in the presence of O_2^- anion was extracted with dioxane (Dorwill, Buenos Aires, Argentina) and the absorbance was measured in a microplate reader at 525 nm (Microplate Reader Benchmark. Bio-Rad). Results were expressed as nanomoles of reduced NBT/10^6 cells and calculated from a standard curve (15).

Chemicals

Chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA); RPMI 1640 medium was purchased from Gibco (San Diego, USA). Pure limonene was also purchased from Sigma, San Diego, USA**.** All drugs were prepared in distilled water. LNMMA and limonene were prepared in absolute ethanol with a resulting concentration of 0.5% ethanol in each reaction well.

Statistical analysis

Data were analyzed by the Student's t test and by one way ANOVA and Dunnett tests. Differences were considered significant when $p \leq .05$ (16).

RESULTS

Proliferation assays and viability studies

To study the mechanism of action of limonene on cell proliferation and nitric oxide production, different inhibitors of the pathways involved in the modulation of cell proliferation were used. It can be seen in Figure 1(a) that limonene exerted antiproliferative action on tumoral cells. This effect is expressed as the percentage of inhibition of cellular proliferation. This percentage was directly related to the concentrations from 20 to 1,000 μg/mL—, as higher was the concentrations more was the percentage of inhibition. To study the effect of inhibitors of the pathway involved in cell proliferation on limonene action, low and high concentrations of limonene were selected. The highest concentration studied was 150 μg/mL, because concentrations over 150 μg/mL principally produced cytotoxic effects related to necrosis. The antiproliferative effect of low concentrations of limonene (40 μg/mL) was blunted significantly by MEK1/2 inhibitor (MEK is an enzyme involved in ERK pathway) or P-38 inhibitor, whereas the antiproliferative effect of high concentrations of limonene (60 and 150 μg/mL) was reversed only by P-38 inhibitor (Figure 1(b)). Moreover, both the P-38 and MEK

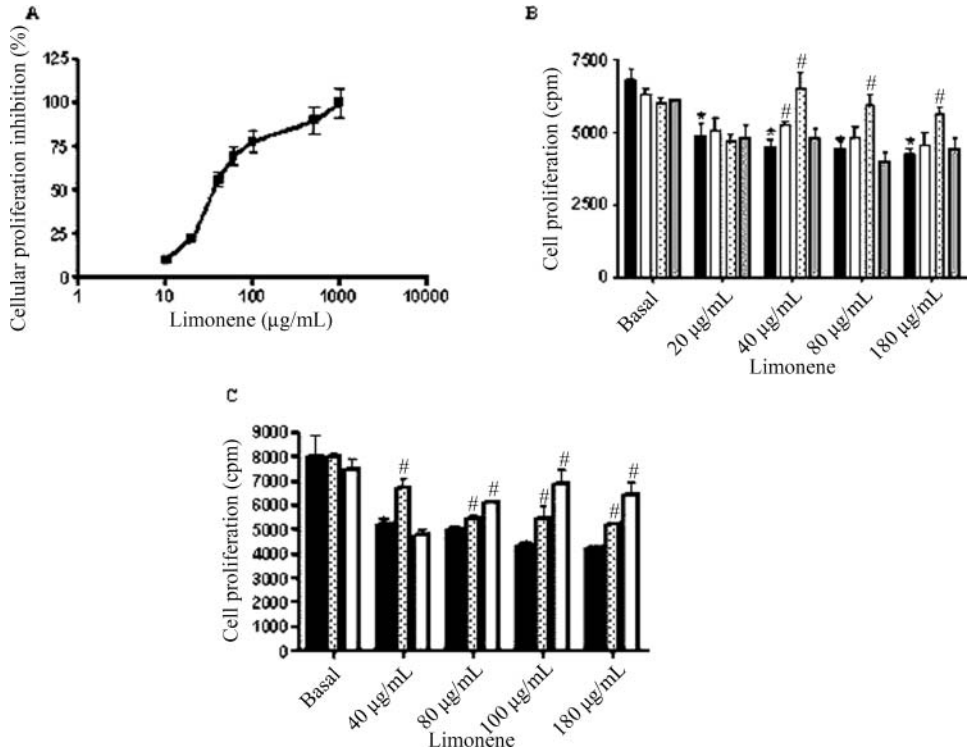

Figure 1. Effect of different concentrations of limonene on cell proliferation (a) and effect of different inhibitors of cell proliferation pathways on limonene action (b) and (c). In (a), limonene was incubated alone. In (b), limonene was incubated alone (black column or in the presence of different inhibitors: Inhibitor of MEK1/2 (white column), inhibitor of P-38 (dotted column), inhibitors of MEK1/2 and P-38 (grey column). In (c), limonene was incubated alone (black column) or in the presence of different inhibitors: L-NMMA (dotted light column), and mevalonic acid (light grey column). Results were expressed as mean ± SEM of three experiments made in triplicate. $^{*}p < .05$ show significant differences with respect to basal value. $^{\#}p < .05$ show significant differences between limonene alone and limonene plus inhibitor treatment, accordingly to Student's *t* test.

inhibitors did not modify the antiproliferative effect of limonene exerted at low or high concentrations.

Moreover, LNMMA blunted the antiproliferative effect of all concentrations of studied limonene; mevalonic acid blunted only the effect of high concentrations of limonene (60, 100, and 150 μg/mL) (see Figure 1(c)). The inhibitors *per se* did not modify basal proliferation.

Apoptosis assays

Taking into account that limonene induced apoptosis in these cells, this phenomenon was studied in tumoral cells treated with limonene alone at low and high concentrations and in presence of MEK1/2 inhibitor, P-38 inhibitor, and mevalonic acid. Depending on the concentration, limonene induced cells to early apoptosis (20 μg/mL), and early and late apoptosis (40 and 60 μg/mL), necrosis was shown with 60 μg/mL and higher concentrations (see Figure 2 and Table 1). A considerable percentage of necrosis and cells in late apoptosis was observed with 150 μg/mL (viable cells 6.18%, early apoptosis 14.26%, late apoptosis 52%, and necrosis 27%). The MEK1/2 and P-38 inhibitors reversed the effect of limonene at 20 and 40 μg/mL, whereas the apoptotic effect exerted by 60 μg/mL limonene was reverted by the P-38 inhibitor and mevalonic acid, thereby increasing the percentage of the viable cells. None of the inhibitors modified the basal parameters. Even so, mevalonic acid alone appeared to produce late apoptosis, nevertheless it did not affect basal cell proliferation.

Total nitrite determination

On the other hand, the production of total nitrites induced by limonene was analyzed in the presence and absence of inhibitors that modulate the proliferative pathways. First, the inhibitors alone did not modify the basal NO level, except MEK inhibitor, which decreased basal NO level (basal value: 0.000012 ± 0.000001 mM; MEK inhibitor: 0.000007 ± 0.0000006 mM). High and low concentrations of limonene significantly increased the total nitrite levels. As can be seen in Figure 3(a), LNMMA reverted the increase of total nitrites exerted by high and low concentrations of limonene, but the MEK inhibitor blunted the effect of low concentrations of limonene. To analyze whether limonene could produce NO by mitochondria, an inhibitor of the transfer of electrons was employed, this inhibitor only reversed the effect of limonene (60 μg/mL). By other way, mevalonic acid blunted the effect of high concentrations of limonene.

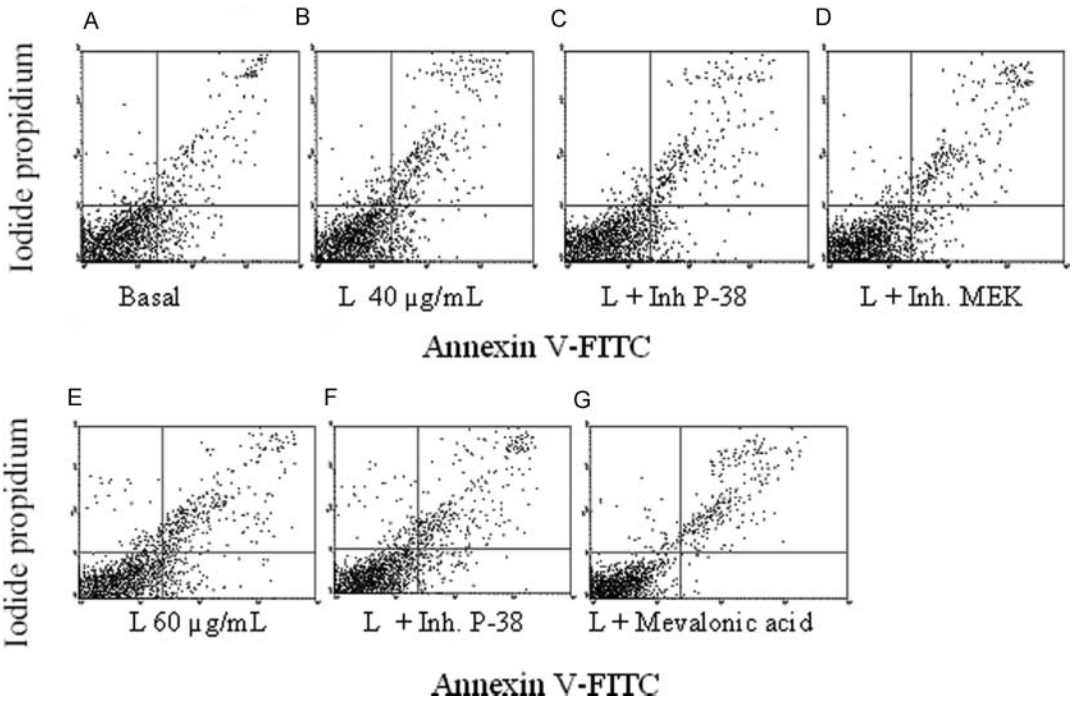

Figure 2. Apoptosis induced by limonene in relation with P-38 and ERK pathways. The figures show representative dot plot analyses of three independent experiments. BW5147 cells were stained with annexin V-FITC and iodide propidium after different treatments. (a) Tumoral untreated cells (basal), (b) treated with limonene 40 μg/mL, (c) limonene 40 μg/mL + inhibitor of P-38, (d) 40 μg/mL limonene + inhibitor of MEK$^1/_2$, (e) 60 μg/mL limonene, (f) 60 μg/mL limonene + inhibitor of P-38, and (g) limonene 60 μg/mL + mevalonic acid. In each figure upper left panel (necrosis), upper right panel (late apoptosis or necrosis), lower left panel (viable cells), and lower right panel (early apoptosis).

NF-kB assays

To relate the increase in NO levels, induced by limonene, to a nuclear induction effect through the activation of NF-κB by ERK pathway activation, the study was performed in the presence and absence of MEK1/2 inhibitor. Limonene increased NF-κB nuclear translocation not only at the concentration of 40 μg/mL but also at 60 μg/mL; but this effect was not reversed by the MEK1/2 inhibitor because it *per se* induced nuclear translocation of NF-κB only (Figure 3(b)).

Table 1. Percentage of Cells at Different Stages of Apoptosis. The Percentage was Calculated from the Dot Plot Analysis

	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis
Basal	81.45	7.44	8.15	2.96
Control apoptosis	**60.25**	**17.48**	**17.95**	**4.33**
Etanol 0.5%	82	7	8.5	2.5
Inh P-38	80.16	10.18	6.53	3.13
Inh MEK	82.17	7.43	8.39	2.02
Mevalonic acid	74.50	8.95	13.97	2.58
Limonene 20 μg/mL	74.07	13.33	9.52	3.07
+Inh P-38	74.94	10.89	11.68	2.49
+Inh MEK	76.30	10.09	11.74	1.87
+Mevalonic acid	71.01	10.37	16.09	2.53
Limonene 40 μg/mL	**70.99**	**11.57**	**14.35**	**3.10**
+Inh P-38	77.14	9.79	10.99	2.08
+Inh MEK	75	8.07	13	3.93
+Mevalonic acid	71.46	7.59	17.36	3.59
Limonene 60 μg/mL	**67.40**	**12.09**	**16.11**	**4.39**
+Inh P-38	73.89	7.14	14.82	4.15
+Inh MEK	68.48	11.10	16.88	3.54
+Mevalonic	81.03	2.41	14.77	1.79

iNOS western blot

To correlate the NF-κB translocation with the induction of iNOS, the effect of this concentration of limonene was studied on iNOS expression. Also, a higher concentration was analyzed. Limonene induced iNOS expression at a short incubation time (4 hr), only at low concentration of 40 μg/mL (see Figure 3(c)), but not at higher concentration of 60 and 100 μg/mL (data not shown). At long incubation time (24 hr), limonene 100 μg/mL increased iNOS expression (Figure 3(c)). By other way, MEK$^1/_2$ inhibitor reversed iNOS expression induced by 40 μg/mL limonene; the western blot is shown in Figure 3(c).

SOD and PER activity and release of H_2O_2

Taking into account that the ERK pathway is known to be related to the production of NO through the activation exerted by H_2O_2, the effect of limonene on cell SOD activity and H_2O_2 levels were studied. Limonene significantly increased H_2O_2 production with respect to basal values at low concentration (40 μg/mL), whereas at higher concentrations (100 μg/mL) it did not not show any change (Table 2(a)), contrarily, the MEK1/2 inhibitor reverted the increase of H_2O_2

Table 2. Effect of Different Concentrations of Limonene on the Metabolism of Reactive Oxygen Species

A

	Hydrogen Peroxide (mM/10^6 cells)		
Treatment	Alone	40 μg/mL Limonene	100 μg/mL Limonene
Basal	0.0578 ± 0.0088	0.085 ± 0.005*	0.0580 ± 0.004
MEK1/2 inhibitor	0.0580 ± 0.0078	0.055 ± 0.0050[&]	0.056 ± 0.005

	Superoxide anion (nmol of reduced NBT/10^6 cells)	
Treatment	Alone	100 μg/mL Limonene
Basal	21.53 ± 0.054	27.11 ± 0.52**
Mevalonic acid	21.53 ± 0.175	18.08 ± 1.49[#]

B

	SOD Activity (U/mL/10^6 cells)		PER activity (U/mL/10^6 cells)	
	1 hr	24 hr	1 hr	24 hr
Basal	0.66 ± 0.060	1.1 ± 0.050	$3 \times 10^{-4} \pm 5 \times 10^{-5}$	$5 \times 10^{-4} \pm 1 \times 10^{-5}$
40 μg/mL Limonene	0.77 ± 0.075*	2.11 ± 0.1**	$1.3 \times 10^{-4} \pm 1 \times 10^{-5}$**	ND
100 μg/mL Limonene	0.20 ± 0.02**	0.31 ± 0.03**	$1.5 \times 10^{-4} \pm 2 \times 10^{-5}$**	ND

Cells were incubated in the presence or absence of 40 /and 100 μg/mL limonene, the enzyme activities were determined after 1 and 24 hr of incubation. ND: not detectable with this method;*$p < .05$; **$p < .01$ show significant differences between basal and treatment values; [#]$p < .05$ show significant differences between limonene alone and limonene + mevalonic acid or limonene + ERK1/2 inhibitor.

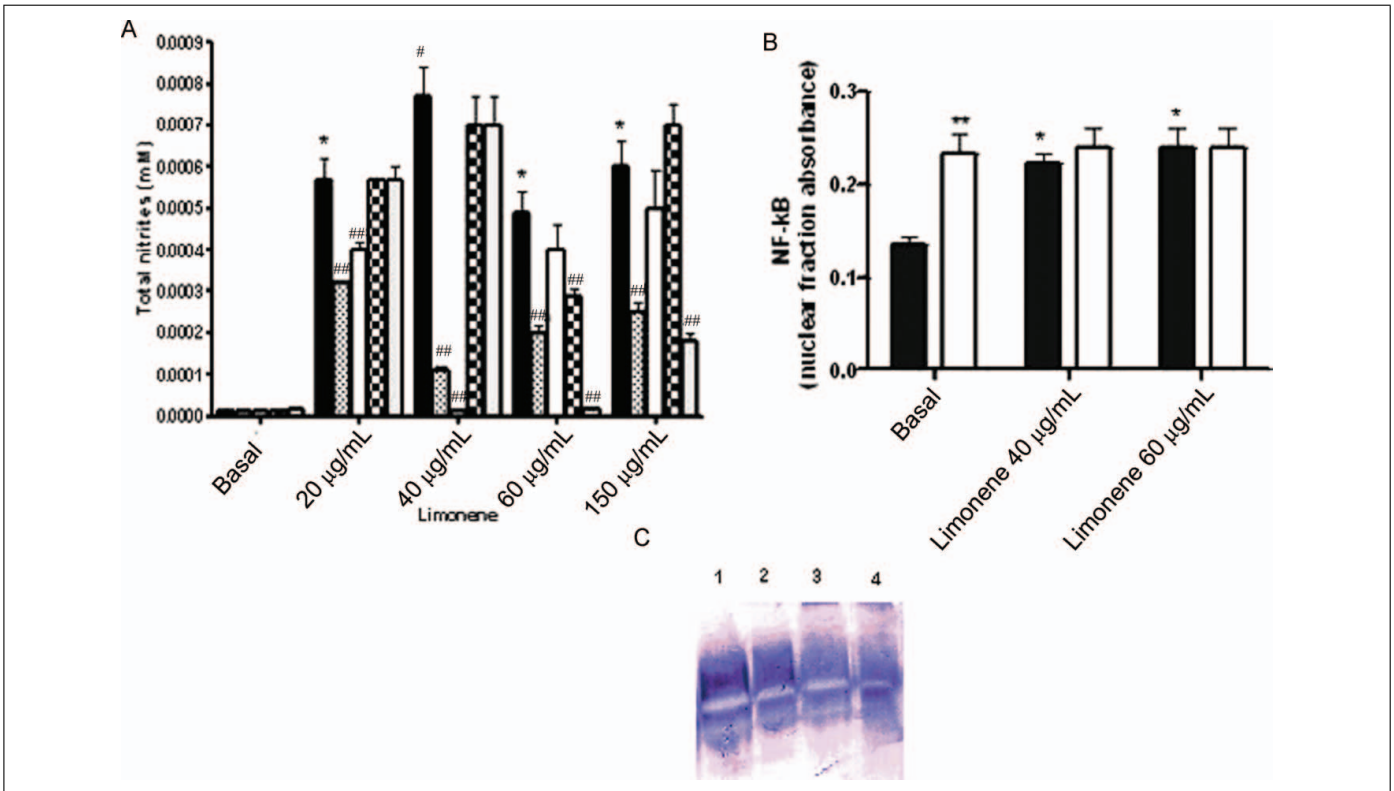

Figure 3. Effect of limonene on total nitrites (a), in relation with NF-κB (b), and iNOS expression (c). In (a), cells were treated with different concentrations of limonene alone (black column) or in the presence of LNMMA (dotted light grey column), MEK1/2 inhibitor (white column), rotenone (white column with black squares), and mevalonic acid (ligth grey column). *$p < .05$ show significant differences between basal values and limonene, [#]$p < .05$ show significant differences between limonene alone and different treatment (ANOVA plus Dunnett's test). In (b), nuclear NF-κB translocation was determined as nuclear absorbance in tumoral cells in the presence of different concentrations of limonene (black column) alone or plus MEK1/2 inhibitor (white column). Cells were incubated during 4 hr. *$p < .05$ show significant differences with respect to basal cells according to ANOVA plus Dunnett's test. (c) is a representative photograph of Western blot analysis of iNOS from three independent experiments. 1: limonene 100 μg/mL at 24 hr of incubation, 2: 40 μg/mL limonene + MEK1/2 inhibitor, 3: 40 μg/mL limonene at 4 hr of incubation, and 4: Basal.

exerted by limonene (Table 2(a)). To evaluate whether the increase in H_2O_2 levels was due to an increase in SOD activity, the activity of the enzyme in these cells was studied in presence of limonene at two times of cell incubation. At 1 hr of incubation, 40 μg/mL limonene significantly increased the basal cell SOD activity but decreased the basal cell peroxidase (PER) activity. At 24 hr, 40 μg/mL limonene significantly increased the basal SOD activity, meanwhile basal cell PER activity could not be detected in the presence of limonene (Table 2(b)). Higher concentrations of limonene (100 μg/mL) decreased cell SOD and PER activities at 1hr and 24 hr (Table 2(b)).

Production of superoxide anion

On the other hand, to corroborate the decrease in SOD activity exerted by 100 μg/mL limonene, the level of superoxide anion was evaluated in the presence of both 100 μg/mL of limonene alone and limonene plus mevalonic acid. It was observed that limonene significantly increased the levels of superoxide anion, whereas mevalonic acid could revert this phenomenon (Table 2(a)).

Finally, to verify whether the effect exerted by 40 μg/mL of limonene was mediated by the activation of SOD, the effect of sodium azide (an inhibitor of the MmSOD) on the decrease of cell proliferation induced by limonene and NO production was studied. Sodium azide *per se* produced a decrease in cell proliferation and diminished NO basal values. Sodium azide could not revert the effect of limonene on cell proliferation but reverted the effect of limonene on NO induction (Figure 4(a) and (b)).

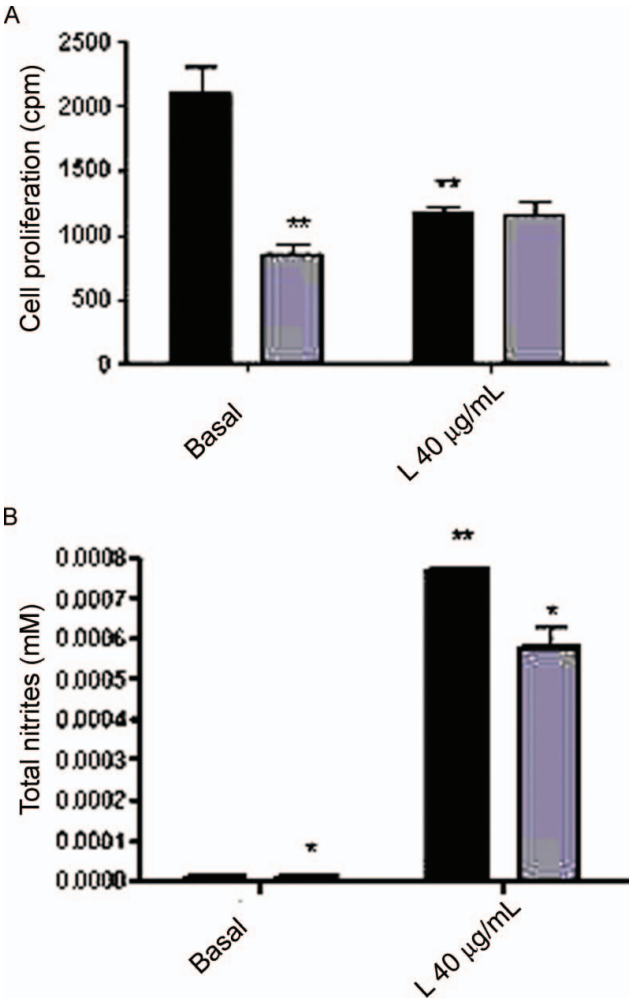

Figure 4. Effect of sodium azide on the response exerted by 40 μg/mL limonene on cell proliferation (a) and on total nitrites (b). Cells were incubated alone (basal), with 40 μg/mL limonene alone (black column), or in the presence of sodium azide (grey column). Results were expressed as mean $\pm$ SEM of three experiments made in triplicate. $^{**}p < .01$ show significant differences with respect to basal value; $^{*}p < .05$ show significant differences between limonene alone and limonene plus sodium azide (Student's *t* test).

DISCUSSION

In this work, the mechanism by which limonene exerted an antiproliferative effect and increased NO levels on BW5147 cells is described by analyzing the pathways related to the cell proliferation.

The antiproliferative activity of limonene on BW5147 cells is known to be produced by exerting a cytostatic and cytotoxic effects, which has been described earlier (4, 5). Limonene is a monoterpene constituent of essential oils of plants. Monoterpenes have been found to have chemopreventive effects on chemically induced mammary gland tumors (17), whereas geraniol, another monoterpene, has exhibited antitumoral activity on leukaemia, hepatoma, and murine melanoma (18). Additionally, limonene has been reported to have a chemopreventive and anticancer effects on several types of tumors in animals. For example, limonene was observed to inhibit the development of spontaneous neoplasmas as well as the development of NMU-induced mammary tumors in mice (2). It has also been reported to reduce the incidence of lymphomas in p53 knockout mice (1) and to produce the regression of NMU- and DMBA-induced mammary tumors (3). The survival of lymphoma (L-5178-Y)-bearing mice fed with a diet rich in limonene was studied by Del Toro-Arreola (19), who observed an increase in the survival time as well as step-up in delayed hypersensitivity reaction to DNFB, phagocytosis, and microbicidal activity.

P-38 MAPK and ERK are known to be redox-dependent biomolecules that modulate cell proliferation, survival, and death. The ERK pathway is mainly activated by mitogenic stimuli, such as growth factors, cytokines, and phorbol esters and plays a major role in regulating cell growth and differentiation (20). It is known that the P-38 MAK activity can be regulated by oxygen reactive species and NO, the latter, in turn, inversely regulates the expression of cyclin D1, which is implicated in G1 progression in hepatocytes and other proliferating cell types (21). Therefore, the P-38 and ERK pathways were studied in relation to the effects observed with limonene on tumoral cell proliferation. The antiproliferative effect of low concentrations of limonene was blunted by MEK1/2 inhibitor and P-38

inhibitor. The fact that MEK1/2 inhibitor reverted the inhibitory effects of limonene on cell proliferation, suggested that this effect was mediated by the activation of the ERK pathway. The inhibitory effect of limonene could also be reverted by the P-38 inhibitor, suggesting a participation of this pathway in its action. As the NOS inhibitor LNMMA reverted the effect of low concentrations of limonene, the participation of NO in its effect can be deduced. Moreover, limonene produced an activation of NOS, as was shown earlier by Manuele *et al*. (5). The fact that both the inhibitors (P-38 and MEK$^1/_2$) did not revert the effect of limonene, could indicate that limonene also exerted its action on another site, just as NOS. When MEK1/2 inhibitor was used, the reversion was about 17%, with P-38 inhibitor, the inhibition was 44% (perhaps this site was the most important one), and when inhibitor of NOS was used, the reversion was 27%. The fact that the reversion produced with each inhibitor was not 100% indicates that limonene presented different sites of action as P-38, MEK$^1/_2$, and NOS that contributed to its antiproliferative action. Nevertheless, when the two inhibitors P-38 and MEK were used together, no reversion was observed, so it could be possible that limonene presented its antiproliferative action through NOS activation, which became the most important site of action under these conditions without P-38 or MEK control. Moreover, it could be possible that limonene had low affinity on NOS, but when P-38 and MEK are inhibited, a hight percentage of limonene exerts its anti-proliferative action mainly by a direct NOS activation bound to NOS exerting activation of the enzyme, exerting in consequence its antiproliferative effect.

By the other hand, the antiproliferative effect of the high concentrations of limonene was blunted by P-38 inhibitor, LNMMA, and mevalonic acid. These results suggested that limonene at high concentrations also acted through a combined mechanisms in which NO and the inhibition of protein farnesylation are involved.

It has been proposed that in some types of tumor cells, limonene induced the inhibition of the farnesylation of p21 Ras protein, thereby inducing a cell cycle arrest and the inhibition of cell proliferation (22, 23). Previously it was observed that LNMMA could revert the antiproliferative effect of low concentrations of limonene as well as the apoptosis and the cell cycle arrest (5).

The antiproliferative effect of limonene was related to the induction of apoptosis. These results are in line with previous works carried out in our laboratory (5). Other authors have also observed that limonene induced apoptosis of hepatic and gastric cells without affecting farnesylation of protein p21 Ras (24, 25). Limonene induced cells to different stages of apoptosis depending on its earlier produced concentration, late apoptosis, or necrosis. The apoptosis induced by low concentrations of limonene was reverted by the MEK1/2 inhibitor as well as the P-38 inhibitor. The blunting effect was also observed on cell proliferation, whereas the apoptosis induced by high concentrations was reverted by the P-38 inhibitor and principally by mevalonic acid. It is important to note that a displacements from necrosis to late apoptosis, from late apoptosis to early apoptosis, and finally from early apoptosis to viable cells were observed in the presence of the inhibitors. These results suggest that low concentrations of limonene could exert cytotoxic effects through the activation of P-38 and ERK pathways. The ERK pathway as well as P38 are not only related to cell proliferation but also to cell death. It is known that the responses of eukaryotic cells to external stimuli are partially regulated by the activation of the three major MAP kinase signaling pathways—kinases 1 and 2 (ERK), Jun N-terminal kinase (JUNK), and P38 MAPK—these kinases are known to mediate many of the cellular processes related to the cell growth, survival, and death. In BW5147 cells, the activation of ERK and P-38 pathways lead to cell death by apoptosis, as it occurs in the presence of high concentrations of H_2O_2 (6). Wang *et al*. (26) observed that the antiproliferative action exerted by plumbagin on human melanoma A 375.S2 cells is mediated by ERK 1/2 activation.

On the other hand, high concentrations of limonene exerted apoptosis through both P-38 pathway and inhibition of protein farnesylation. It was shown that the treatment of four medulloblastoma cell lines *in vitro* with manumycin A, an antibiotic, which inhibits farnesyl protein transferase and thus proteins farnesylation, induces antiproliferative effects and apoptosis in a time- and dose-dependent manner (27). Moreover, the farnesyl transferase inhibitor FTI-277 inhibits cell growth and induces apoptosis in drug-resistant myeloma tumor cells (28). Inhibition of Ha–Ras farnesylation prevents the protein from binding to the plasma membrane and leads to the cytosolic sequestration of inactivated c-Raf-1 and consequently to the inhibition of cell proliferation (29). An oncogenic Ras is incapable of transforming cells without farnesylation (30). Therefore, as we have seen with limonene, other inhibitors of protein farnesylation, such as lovastatin, induce apoptosis by the activation of p38 mitogen-activated protein kinase, and by the inhibition of ERK pathway along with the activation of NF-κB. The latter pathways are activated by ROS, indicating that cell death induced by lovastatin is dependent on oxidative stress (31). Nevertheless, mevalonic acid by itself appeared to produce apoptosis did not modify cell proliferation.

It is known that the MEK/ERK pathway is related to the NO production and that NO in turn induces apoptosis by the activation of the P-38 pathway. Besides, NO can directly induce cytochrome-c release through the potential loss of mitochondrial membrane leading to cell death by apoptosis via the activation of the JNK/SAP and P-38 MAPK pathways (32, 33). Moreover, the cytotoxic effects of NO on some tumor cells are due to the accumulation of P53 and the increase in Bax/Bcl-Xl, both effects leading to DNA damage (34).

Then the effect of limonene on NO was analyzed alone and in presence of the inhibitors of the pathways involved in cell proliferation. Limonene produced an increase in NO level in all concentrations studied. The increase of NO exerted by low concentrations was reverted by MEK1/2 inhibitor and LNMMA, whereas the effect induced by high concentrations was reverted by LNMMA, mevalonic acid, and the mitochondrial chain respiratory inhibitor rotenone. The increase in NO levels produced by low concentrations of limonene has been observed earlier also (5). In agreement with our results, it is shown that

the activation of ERK and P-38 pathways is related to death via production of NO (35). Moreover, it is a well-known fact that the ERK pathway is related to NO production through the activation exerted by H_2O_2, which in turn induces NF-κB translocation (6). The production of NO, through the activation of the ERK pathway, induced by low concentrations of limonene was mediated by NF-κB nuclear translocation. This effect, which is in line with the results obtained by Davicino (6), was probably related to the increase in iNOS expression observed by us. Moreover, high concentrations of limonene also increased NF-κB nuclear translocation and iNOS expression. Nevertheless, the MEK1/2 inhibitor did not revert these effects, probably this was related to the fact that MEK1/2 inhibitor *per se* increased the basal NF-κB translocation. The increased translocation of NF-κB exerted by MEK1/2 inhibitor *per se* could be related to the decrease of NO level detected in cell culture. It was shown that NO can regulate NF-κB both positively and negatively at multiple steps in the activating pathway, depending on the cell type, cell stimulus, NO concentrations, and NO-related species. On the other hand, inhibitors of NO synthase, which conduce to a decrease in NO level, augment NF-κB activity in resting endothelial cells and LPS-stimulated macrophages (6, 36–38). Also, NO increases the transcription and stability of the NF-κB inhibitory protein, IκBα, therefore, NF-κB can not activate. Moreover, it was reported that NO can induce modification in NF-κB molecules, preventing binding to its target DNA site (36, 39). Furthermore, it was shown that MEK inhibitors, as PD 98059, influences the post-transcriptional events involved in iNOS activation, causing a decrease in nitrite production. Hence, the MEK inhibitor used in this study could act as an iNOS inhibitor, which can explain the low level of NO observed in the presence of MEK inhibitor (40).

The fact that the inhibitor of transfer of electrons reverted the increase of NO levels induced by high concentrations of limonene suggests that limonene could act on mitochondrial production of NO. Different mitochondrial NO synthase (mt-NOS) isoforms have been described in rat and mouse tissues, such as liver, thymus, skeletal muscle, and, more recently, heart and brain (41). On the other hand, farnesylation of proteins was observed to be implicated in the modulation of NO, as mevalonic acid could revert the increase in NO levels induced by high concentrations of limonene. Besides, it is known that mevalonic acid can also revert the upregulation of eNOS induced by lovastatin, an *in vivo* inhibitor of farnesylation of proteins (42). Moreover, in human malignant mesothelioma (HMM), which is resistant to many anticancer drugs, such as doxorubicin, mevastatin, and simvastatin, two inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase (enzymes that inhibits isoprenoids formation and consequently proteins farnesylation) enhanced the intracellular accumulation and the cytotoxicity of doxorubicin, essentially expressing P-glycoprotein and multidrug resistance-associated protein 3. This effect of statins was NO dependent, because it was reverted by either an NOS inhibitor or an NO-scavenging system. The upregulation of NOS in HMM and other cells is known to be associated with the activation of the transcription factor NF-κB in HMM cell statins, thus increasing the NF-κB translocation into the nucleus and decreasing the level of the NF-κB inhibitor (43). Taking into account that the increase in NO levels produced by low concentrations of limonene was related to the induction of iNOS expression, this phenomenon could be related to a previous production of H_2O_2, which in turn could be implicated in iNOS induction, and the effect of limonene on H_2O_2 production was analyzed. Low concentrations of limonene produced a significant increase in H_2O_2 level, but high concentrations did not show such effects. The increase in H_2O_2 level produced by limonene at low concentrations was principally related to the decrease in cell PER activity (58% of decrease), enzyme that is known to be associated with the destruction of H_2O_2; limonene at low concentrations increased SOD activation (enzyme related to H_2O_2 formation), the increase being only about 16% at 1 hr of incubation. Nevertheless, it is probable that limonene in low concentrations can increase H_2O_2 after 24 hr of incubation, as it significantly increased basal cell SOD activity and diminished basal PER activity at values not detected with the employed method. On the other hand, high concentrations of limonene did not increase H_2O_2 levels, this fact is related with the decrease in basal SOD activity exerted by limonene at 1 and 24 hr of incubation. The increase in SOD activity exerted by low concentrations of limonene could be related to the activation of the ERK pathway that induces MnSOD expression (and increase in H_2O_2 level). Conversely, the ERK pathway inhibition as exerted by the high concentrations of limonene that induced a decrease in basal SOD activity can be related to a decrease in MnSOD expression, as was observed with lovastatin, an inhibitor of Ras protein farnesylation (31). The H_2O_2 induced by the low concentration of limonene derived from ERK pathway as MEK1/2 inhibitor reverted this effect. The activation of the ERK pathway by oxidative stress is reported in a variety of cell types (44).

The inhibition of SOD activity exerted by high concentrations of limonene is related to an increase in superoxide anion, which in turn provoked oxidative stress and the formation of other free radicals of NO.

It was observed that some natural products such as plumbagin have antiproliferative effects and induce apoptosis on human melanoma A375.S2 cell line by increasing the production of O_2^- and H_2O_2 (26). Moreover, the inhibition of O_2^- by sacavenger p-benzoquinone prevents camptothecin-induced apoptosis (45). It is well known that cell survival depends on ROS intracellular levels (46), the increase in ROS can produce an alteration of the mitochondrial membrane permeability and cell apoptosis (47).

To sum up, limonene, depending on concentration, was capable of increasing NO levels and produced antiproliferative effects by inducing cells to apoptosis by two mechanisms. It could be deduced that limonene produced all these effects by a dual action on ERK pathways: at low concentrations it activated the production of H_2O_2, which in turn activated ERK pathway, intracellular signals related with the increase in NO level; at high concentrations, the inhibition of farnesylation of proteins appeared to be the most important effect involved in the increase of NO levels exerted by limonene, probably by NOS activation. It is important to note that both mechanisms of activation and

inhibition of the ERK pathway were related to the increase in total nitrites by different mechanisms where the oxidative stress was implicated.

DECLARATION OF INTEREST

There are no conflicts of interest.

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