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Activity of *Melaleuca alternifolia* (tea tree) oil on Influenza virus A/PR/8: Study on the mechanism of action

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ABSTRACT

Our previous study demonstrated that *Melaleuca alternifolia* (tea tree) oil (TTO) had an interesting antiviral activity against Influenza A in MDCK cells. In fact, when we tested TTO and some of its components, we found that TTO had an inhibitory effect on influenza virus replication at doses below the cytotoxic dose; terpinen-4-ol, terpinolene, and alfa-terpineol were the main active components.

The aim of this study was to investigate the mechanism of action of TTO and its active components against Influenza A/PR/8 virus subtype H1N1 in MDCK cells.

None of the test compounds showed virucidal activity nor any protective action for the MDCK cells. Thus, the effect of TTO and its active components on different steps of the replicative cycle of influenza virus was studied by adding the test compounds at various times after infection. These experiments revealed that viral replication was significantly inhibited if TTO was added within 2 h of infection, indicating an interference with an early step of the viral replicative cycle of influenza virus.

The influence of the compound on the virus adsorption step, studied by the infective center assay, indicated that TTO did not interfere with cellular attachment of the virus.

TTO did not inhibit influenza virus neuraminidase activity, as shown by the experiment measuring the amount of 4-methylumbelliferone, cleaved by the influenza virus neuraminidase from the fluorogenic substrate 2′-*O*-(4-methylumbelliferyl)-*N*-acetylneuraminic acid.

The effect of TTO on acidification of cellular lysosomes was studied by vital staining with acridine orange using bafilomycin A1 as positive control. The treatment of cells with 0.01% (v/v) of TTO at 37 °C for 4 h before staining inhibited the acridine orange accumulation in acid cytoplasmic vesicles, indicating that TTO could inhibit viral uncoating by an interference with acidification of intralysosomal compartment.

1. Introduction

Over the last few years there has been an increasing interest in the anti-microbial properties of tea tree oil (TTO), known for a long time as a general antiseptic. TTO is extracted from the leaves and terminal branches of *Melaleuca alternifolia*, also called the tea tree. *M. alternifolia* grows in a restricted marshy area of northern New South Wales, in Australia.

TTO is a heterogeneous mixture, subject to considerable batch-to-batch variation depending on growth conditions at the plantations (Kawakami et al., 1990; Shellie et al., 2003). The exact constituency of tea tree oil varies, as well as the antibacterial, antifungal, anti-inflammatory and analgesic properties (Caldefie-Chézet et al., 2006; Carson and Riley, 1993; Carson et al., 2006; Hammer et al., 2004; Hart et al., 2000; Wilkinson and Cavanagh, 2005).

Our interest in natural compounds with antiviral activity led us to analyze TTO against DNA and RNA viruses (Influenza A/PR/8 subtype H1N1, polio type 1, ECHO 9, Coxsackie B1, adeno type 2, herpes simplex (HSV) type 1 and 2 viruses). Our results demonstrated that TTO and some of its components (terpinen-4-ol, terpinolene, and α-terpineol) have an inhibitory effect on Influenza A/PR/8 virus subtype H1N1 replication at doses below the cytotoxic dose. The EC_{50} value of TTO was found to be 0.0006% (v/v) and was much lower than its CC_{50} (0.025%, v/v). All the compounds were ineffective against polio 1, adeno 2, ECHO 9, Coxsackie B1, HSV-1, and HSV-2 (Garozzo et al., 2009). This was in contrast with the results reported by other authors who demonstrated antiviral activity against the replicative cycle of HSV-1 and HSV-2 (Carson et al., 2001, 2008; Schnitzler et al., 2001).

The aim of the present study was to determine the mechanism of action of TTO and its active components, terpinen-4-ol, terpinolene and α-terpineol, (test compounds) against Influenza A/PR/8 virus subtype H1N1.

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2. Materials and methods

2.1. Compounds

The essential oil of *M. alternifolia* was provided by Australian Botanical Products (Hallam, Australia). Terpinen-4-ol, terpinolene and α-terpineol were obtained from Sigma Chemical Company. All the compounds were initially dissolved in DMSO (dimethyl sulfoxide, Sigma) to a concentration of 5% (v/v).

Oseltamivir and ribavirin were used as reference compounds: oseltamivir phosphate was used directly from the blister packaging of Tamiflu 75 mg (Roche Product) and prepared in sterile glass-distilled water supplemented with 12.5% (v/v) DMSO to a concentration of 1 mM; ribavirin (1-p-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was purchased from Sigma Chemical Company. Ribavirin was prepared in sterile glass-distilled water to a concentration of 2 mM.

The macrolide antibiotic bafilomycin A1 (Sigma) was used as reference compound blocking the vacuolar H^+-ATPase proton pump (Yoshimori et al., 1991). The drug was prepared in DMSO at 10 μM.

Acridine orange (AO) (Sigma) was prepared in sterile glass-distilled water at 50 mg/ml.

All the compounds were diluted in maintenance medium before use to achieve the final concentration needed. The dilution of the test compounds contained a maximum concentration of 0.01% DMSO, which was not toxic to the cells.

2.2. Cells and virus

Madin–Darby canine kidney (MDCK) cells were kept in a humidified 5% carbon dioxide atmosphere at 37 °C and grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 0.1% sodium bicarbonate, 200 μg/ml of streptomycin and 200 units/ml of penicillin G.

Influenza virus A/Puerto Rico/8/34 H1N1 (PR8) kindly supplied by Prof. L. Nencioni (Università di Roma La Sapienza, Rome, Italy), was grown in MDCK cells at 37 °C.

The working stock solution was prepared as cellular lysates using RPMI 1640 with 2% FCS (maintenance medium) and centrifuged (5000 × *g* for 30 min at 4 °C).

Infectivity of virus stock and virus yield in the experiments on the mechanism of action were determined by the MTT method: the reciprocals of viral dilution which resulted in 50% reduction of absorbance of formazan in the infected cells at 48–72 h was determined as infectivity of the virus by MTT ID_{50} (50% infective dose) (Garozzo et al., 2000).

Virus stock solution was also used as a source of neuraminidase (NA) in the NA inhibition assay.

Virus yield was also evaluated by measuring haemagglutinin units (HAU). Briefly, twofold serial dilution of each test was mixed with an equal volume (50 μl) of 0.5% suspension of human red blood cells of the O Rh+ group and incubated for 30 min at room temperature.

The viral titre of the sample was the reciprocal of the dilution giving rise to complete haemoagglutination.

2.3. Virucidal activity

To test possible virucidal activity, equal volumes (0.5 ml) of influenza virus suspension, containing 10 $CCID_{50}$ (50% cell culture infective dose), and RPMI containing the various concentrations of the test compounds were mixed and incubated for 2 h at 37 °C. Infectivity was determined as described above after dilution of the virus below the inhibitory concentration.

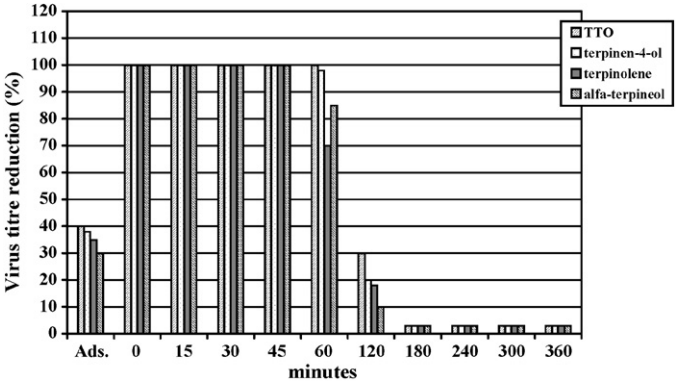

Fig. 1. Effect of the addition of TTO (0.01%, v/v) and its active components, terpinen-4-ol (0.01%, v/v), terpinolene (0.005%, v/v) and α-terpineol (0.02%, v/v) at various times during the replicative cycle of influenza virus. Time 0 = post 2 h adsorption period at 4 °C. Each value represents the mean ± S.E.M. of three separate assays.

2.4. Cell culture pretreatment

Pretreatment of cultures was performed by exposing the cell monolayers to different concentrations of the test compounds in maintenance medium for 1 and 2 days at 37 °C. After treatment the cell monolayers were washed thoroughly with PBS and infected with influenza virus at an MOI (multiplicity of infection) of 0.1 to allow viral cytopathic activity. The cell monolayers grown in maintenance medium without the test compounds were used as controls. Virus titration was performed as described above.

2.5. Addition at different time intervals

Monolayers of MDCK cells were grown to confluence in 24-well plates and inoculated with influenza virus at an MOI of 0.1. The plates were incubated for 2 h at 4 °C to ensure synchronous replication of the viruses, with or without the test compounds for the adsorption period. The inoculum was then removed and the medium, with or without the test compounds, was added at various times after the adsorption period, as indicated in Fig. 1. The plates were incubated at 37 °C for 12 h, the cultures were then frozen and the virus yield was determined as previously described.

2.6. Inhibition of virus attachment assay

The infective center assay was used to study the effect of the test compounds on the virus adsorption step. Briefly, a MDCK cell suspension (10^4 cells/ml) was cooled to 4 °C for at least 1 h. Influenza virus (MOI = 0.1) was incubated for 60 min at 37 °C with different concentrations of the test compounds, cooled to 4 °C, and subsequently added to the cell suspension. Cells were incubated with the virus-compound mixtures for 120 min at 4 °C to prevent the virus from entering the cells. After the adsorption period, unadsorbed virus and free compound were removed by washing three times with cold RPMI. The cells were then diluted serially and assayed for cell-associated viral cytopathic activity as previously described.

2.7. Haemoagglutination inhibition assay

Haemoagglutination inhibition assay was used to test the effect of the test compounds on virus adsorption to target cells. Twenty-five μl two-fold dilution in PBS of the test compounds with equal volumes of influenza virus solution (200 HAU/25 μl) were mixed and incubated for 30 min at room temperature. Fifty μl of 0.5% suspension of human red blood cells of the O Rh+ group was then added and incubated for 30 min at room temperature. The minimum concentration of the compounds that

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completely inhibited the haemoagglutination of the virus was determined.

2.8. Neuraminidase inhibition assay

Influenza virus neuraminidase activity was measured by a modification of the method described by Potier et al. (1979). The assay measures the amount of 4-methylumbelliferone (FL-4-MU) that is cleaved by the influenza virus neuraminidase (NA) from the fluorogenic substrate 2′-*O*-(4-methylumbelliferyl)-*N*-acetylneuraminic acid (FL-4-MU-NANA; Sigma, St. Louis, MO).

Influenza virus NA activity was determined before it was used in NA inhibition tests. Briefly, 75 μM FL-4-MU-NANA were used as substrate in an enzyme buffer consisting of 33 mM 2-(*N*-Morpholino)ethanesulfonic acid (MES; Sigma, St. Louis, MO), 4 mM $CaCl_2$ and pH adjusted to 6.5. Fifty μl of the substrate with 50 μl two-fold dilutions of virus stock were mixed and incubated for 1 h at 37 °C with shaking in the dark. The reaction was stopped by the addition of 150 μl of a stop solution (0.014 M NaOH in 83% ethanol, pH 10.7).

FL-4-MU was immediately quantified in RFUs (relative fluorescence units) using a Wallac Victor 2 multilabel counter with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Wells were read at a rate of 1.0 s per well.

Various volumes (5, 10, 20, and 40 μl) of a 40 μM solution of free FL-4-MU (Sigma, St. Louis, MO) in 0.9% (w/v) NaCl were used in parallel to determine the linear range of detection.

The concentration of virus versus NA activity was plotted graphically to determine the virus/enzyme concentration to be used in subsequent inhibition assays.

NA inhibition assay was used to determine the test compounds concentration required to reduce NA activity by 50% (IC_{50}) (Buxton et al., 2000; Gubareva et al., 2002). Equal volumes (25 μl) of twofold dilutions of test compounds and virus (10,000 RFUs) were mixed and preincubated at 37 °C for 60 min to allow interaction of drug and virus.

50 μl of substrate FL-4-MU-NANA (75 μM) in enzyme buffer pH 6.5 was added, incubated at 37 °C for 60 min under shaking and stopped as previously described.

RFUs of FL-4-MU were immediately quantified, and the percentage of fluorescence inhibition of each compound was calculated by the following formula: $RFU_{virus} - RFU_{test}/RFU_{virus} \times 100$ whereby RFU_{virus} and RFU_{test} indicated the RFU of the virus-infected-control (no compound) and the virus treated with the compound, respectively.

Experimental RFU values were corrected by subtracting the mean RFU value for the background fluorescence (which was a combination of the plate, buffer fluorescence, and mock-infected control).

The data were plotted as compound concentrations against fluorescence inhibition, and the IC_{50s} were read from the graph.

In all the experiments, oseltamivir and ribavirin were used as positive and negative compounds, respectively.

2.9. Effect of TTO on endosomal/lysosomal pH measured by acridine orange accumulation

The effect of TTO on acidification of cellular lysosomes was studied by vital staining with acridine orange (AO) using microscopy and fluorometry.

Vital fluorescence microscopy: The living cultured cells were stained with AO following the method previously described (Geisow et al., 1981; Yoshimori et al., 1991). Briefly, MDCK cells, grown to confluence on sterilized glass coverslips placed in 24-well plates, were incubated at 37 °C for 1, 2, 4 and 24 h with or without the TTO compound (0.01, 0.005, and 0.0025%, v/v) in RPMI medium without the addition of fetal calf serum. At the end of the incubation period the cells were treated with AO (diluted in sterile glass-distilled water to a concentration of 25 μg/ml) at 37 °C for 30 min under 5% CO_2, then washed four times with warm PBS and dried. The coverslips were mounted with cells facing down onto a slide containing a drop of PBS and immediately examined using a Leica DM LB fluorescence light microscope. All steps were carried out in the dark. Photographs of the microscopic observations were taken with a Leica DC180 digital camera system.

Fluorometry: The measurement of fluorescence intensity was used to identify the accumulation of protonated acridine orange in the acid compartment by fluorescence emission (Natale and McCullough, 1998; Zoccarato et al., 1999).

Subconfluent monolayers of MDCK cells, grown in 96-well tissue culture plates, were incubated at 37 °C for 1, 2, 4, and 24 h with or without 100 μl of TTO (0.01, 0.005, and 0.0025%, v/v). Plates were gently shaken on a mechanical vibrator after the addition of 10 μl per well of freshly prepared 10× concentrate solution of AO (100 μg/ml) in RPMI medium and were then incubated for 30 min at 37 °C in the dark. The supernatant was carefully removed and discarded, the cells were washed four times with warm PBS and immediately examined. The fluorescence was quantified in RFUs using a 96-well plate-based spectrofluorometer (Wallac Victor^2 1420-Multilabel Counter, Perkin-Elmer) at λ_{ex} 490 nm and λ_{em} 530 nm. Wells were read at a rate of 1.0 s per well.

In all the experiments, cells incubated at 37 °C for 1 h with bafilomycin A1 at doses (100, 10 and 1 nM) that did not affect MDCK cell viability after a 24 h incubation period were used as positive

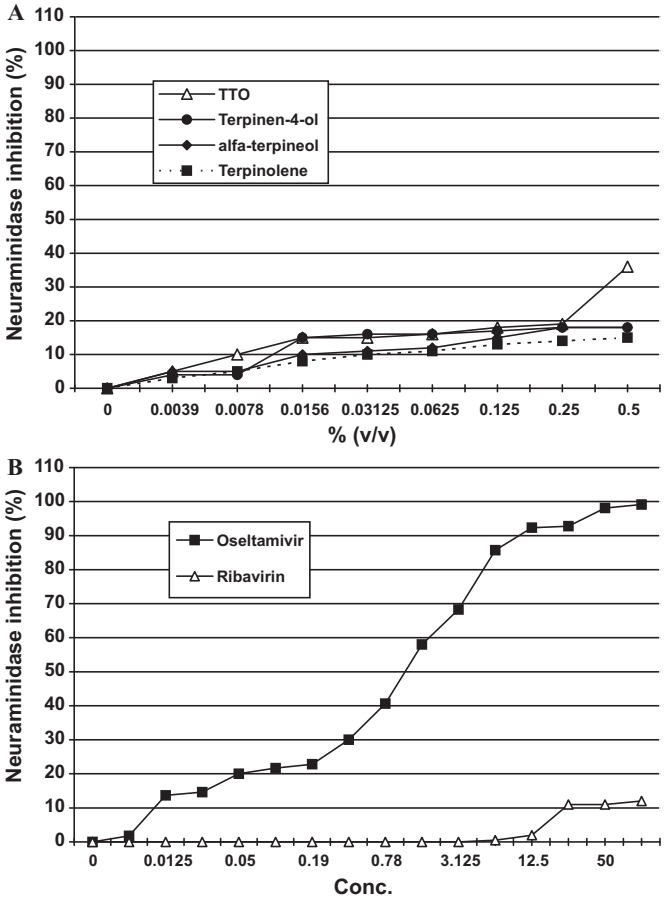

Fig. 2. Effects of TTO and its active components (A), and effects of positive control oseltamivir (nM) and negative control ribavirin (μM) (B) on the influenza virus neuraminidase activity. Values <50% were not considered positive.

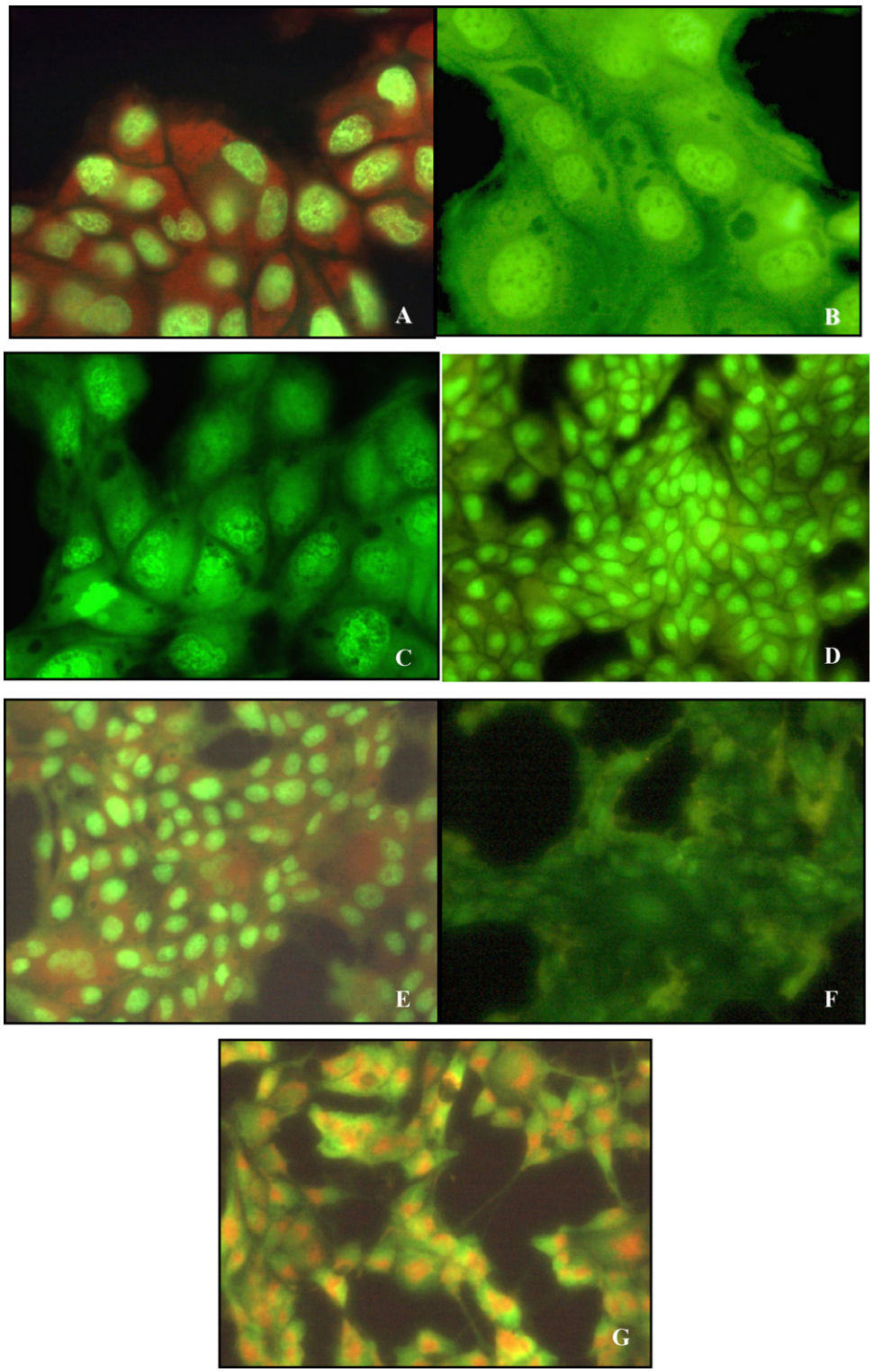

Fig. 3. Acridine orange staining of MDCK cells: untreated (DMSO) (A), treated with 100 and 10 nM bafilomycin A1 for 1 h (B and C); with 0.01% TTO for 4 h (D) and for 1 h (E); with 0.01% terpinen-4-ol for 4 h (F); treated with 0.01% TTO for 4 h followed by washing and incubation without TTO (G).

controls. For control experiments, only DMSO was added to the medium instead of the compounds.

The compounds terpinen-4-ol, terpinolene, and α-terpineol were used at the concentration of 0.01% (v/v), 0.005% (v/v), and 0.02% (v/v), respectively.

The fluorescence signal depends on the AO accumulation in acidified cellular compartments, therefore an increased fluorescence was indicative of a diminished concentration of AO in the cells.

2.10. Reversibility of drug effect

To study the possible reversibility of the TTO effect, MDCK cells were incubated at 37 °C for 4 h with TTO, and terpinen-4-ol (0.01,

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0.005 and 0.0025%, v/v), with bafilomycin A1 (100, 10 and 1 nM) for 1 h or without compounds. After three washings with RPMI, cells were incubated with fresh medium at 37 °C for 2 h and were stained with AO (10 μg/ml) following the method described above and immediately examined and quantified in RFUs.

3. Results and discussion

Recent studies performed in our laboratory revealed that TTO, terpinen-4-ol, terpinolene, and α-terpineol had an inhibitory effect on Influenza A/PR8 virus replication. In fact, TTO had no adverse effect on MDCK cell cultures up to a concentration of 0.025% (v/v), which is much higher than the 50% inhibitory concentration of influenza virus (EC_{50}: 0.0006%, v/v). Three of the TTO components tested were effective. In particular, the EC_{50} values were found to be 0.002% (v/v), 0.025% (v/v) and 0.00125% (v/v) for terpinen-4-ol, α-terpineol, and terpinolene, respectively. Compounds α-terpinene, *p*-cymene and γ-terpinene were completely ineffective (Garozzo et al., 2009).

In the present study, we investigated the antiviral mechanism of action of TTO and its active components against Influenza A/PR/8 virus subtype H1N1.

In all the experiments, influenza virus replication was evaluated in MDCK cells 48–72 h after infection by HAU/ml and CPE_{50} assays. The results of the two methods were consistently concordant, indicating that all observed changes pertained to both viral HA production and infectivity.

In order to determine whether TTO and its components inhibited viral replication at a specific step in the virus cycle, the effect of compound addition at different time intervals using MDCK cells was studied. The results demonstrated that TTO, terpinen-4-ol, terpinolene, and α-terpineol interfere with an early step of the viral replicative cycle of influenza virus. In fact, the test compounds were effective when added within 1 h after the end of the adsorption period while no reduction was observed if they were added more than 2 h after virus adsorption. Only a slight reduction of virus yield was observed if the test compounds were added during the adsorption period and at 120 min (Fig. 1).

Since the period of the most sensitivity of the compounds was an early event in influenza virus replication, it was important to establish whether our compounds produced a virucidal effect or a protective action for the MDCK cells. The results demonstrated that TTO and its active components were not virucidal for influenza virus and did not exert any protective action for the cells (data not shown).

The effect of TTO and its active components under different experimental conditions was studied in order to understand the mechanism of inhibition.

The influence of the test compounds on the virus adsorption step, studied by infective center assays and by haemoagglutination inhibition assay, demonstrated that TTO, α-terpineol, terpinen-4-ol and terpinolene did not interfere with cellular attachment of the virus or with the viral adsorption to RBC resulting in haemagglutination (data not shown). Therefore, the slight reduction of virus yield observed during the adsorption period in the time of addition experiments was, probably, due to residual compounds that could interfere with the following steps.

The ability of TTO and its active components to inhibit the enzymatic activity of viral neuraminidase was tested and compared to that of the known neuraminidase inhibitor oseltamivir (IC_{50} value: 1.34 nM, in our experimental condition) (Hayden et al., 1999).

As shown in Fig. 2A TTO and its active components did not have any inhibitory effect on the influenza virus neuraminidase activity. In fact, the results indicated that there was a substantial difference in the percentage of enzymatic inhibition compared with the positive control oseltamivir (Fig. 2B). Only a slight effect (~35%) was observed at a concentration of 0.5% (v/v) TTO, that was not considered inhibitory (<50%).

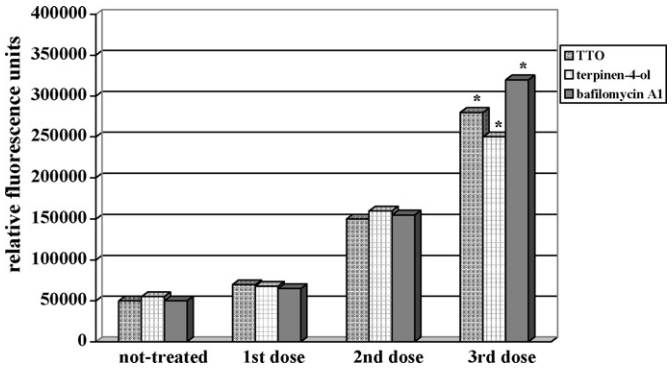

Fig. 4. Fluorescence signal (RFUs: relative fluorescence units) of MDCK cells incubated without compounds, with various concentrations of TTO, and terpinen-4-ol for 4 h at 37 °C, or bafilomycin A1 for 1 h at 37 °C, before treatment with acridine orange. TTO and terpinen-4-ol: 1st dose = 0.0025% (v/v); 2nd dose = 0.005% (v/v); 3rd dose = 0.01% (v/v). Bafilomycin: 1st dose = 1 nM; 2nd dose = 10 nM; 3rd dose = 100 nM. Each value represents the mean ± S.E.M. of three separate assays. The asterisk indicates a significant difference between test samples, $^*P < 0.05$.

Ribavirin was also evaluated in parallel, it is an influenza virus inhibitor with different modes of action unrelated to inhibition of viral neuraminidase (Eriksson et al., 1977; Wray et al., 1985).

It has been demonstrated that the acidic condition in endosomes and lysosomes is essential for the uncoating process of influenza virus infection since it causes the viral envelope fusion activity (Guinea and Carrasco, 1995). Thus, we set up some experiments in order to verify if TTO and its components exerted an inhibitory effect on the acidification of intracellular compartments such as endosomes and lysosome inhibiting the influenza virus growth in MDCK cells.

The effect of the compounds on acidification of lysosomes was tested by vital staining with acridine orange (AO). This dye is a weak base, which is taken up by living cells and protonated in endosomes/lysosomes, resulting in an accumulation of the AO therein.

As a consequence, when untreated MDCK cells were stained with AO, nuclei, nucleoli and the cytoplasm, showed green fluorescence, whereas the granular pattern in the cytoplasm showed orange fluorescence, due to acidified lysosomes (Fig. 3A).

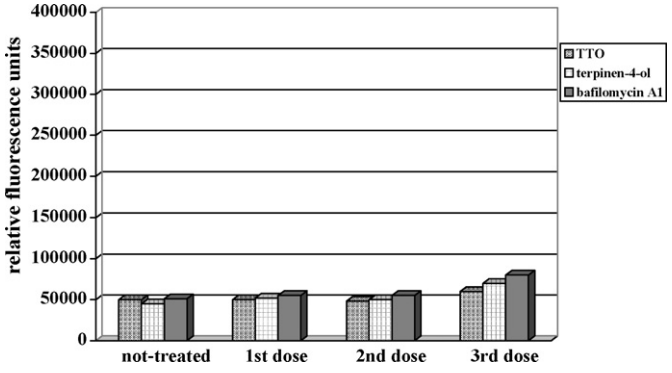

Fig. 5. Reversibility of drug effect: MDCK cells incubated without compounds, with various concentrations of TTO, and terpinen-4-ol for 4 h at 37 °C or bafilomycin A1 for 1 h at 37 °C, washed and incubated without compounds for 2 h at 37 °C, before treatment with acridine orange. TTO and terpinen-4-ol: 1st dose = 0.0025% (v/v); 2nd dose = 0.005% (v/v); 3rd dose = 0.01% (v/v). Bafilomycin: 1st dose = 1 nM; 2nd dose = 10 nM; 3rd dose = 100 nM. Each value represents the mean ± S.E.M. of three separate assays.

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The treatment of the cells with 0.01% (v/v) TTO at 37 °C for 2 and 4 h before acridine orange-staining caused complete disappearance of the orange fluorescence, in contrast the green fluorescence remained (Fig. 3D). The same results were observed if the cells were treated with 100 and 10 nM of bafilomycin A1 at 37 °C for 1 h (Fig. 3B and C).

The treatment of the cells with TTO at the same concentration for 1 h or with a lower concentration (0.0025%, v/v), did not cause the disappearance of the cytoplasmic orange fluorescence (Fig. 3E).

The data obtained by treating the cells with terpinen-4-ol, terpinolene, and α-terpineol before AO staining demonstrated that terpinen-4-ol, one of the major components of TTO, had an important role in the anti influenza virus activity as only in the cells treated with this compound did we observe a lack of the orange fluorescence (Fig. 3F).

These results were confirmed by measuring the fluorescence intensity by fluorometry, indicating that TTO and terpinen-4-ol clearly inhibited acridine orange accumulation in acid cytoplasmic vesicles, in fact, the data obtained were consistently concordant with the positive control bafilomycin A1 (Fig. 4).

The acidification of lysosomes in MDCK cells recovered completely when the cells were treated with 0.01% (v/v) TTO for 4 h, washed and then incubated for 2 h without the compound (Figs. 3G and 5), suggesting that the cells can re-acidify after treatment with TTO, and that cell morphology is not influenced by treatment. A similar result was observed for terpinen-4-ol (Fig. 5).

Our results indicate that TTO inhibited the influenza virus growth in MDCK cells by an interference with acidification of the intralysosomal compartment that could inhibit viral uncoating.

Other studies also reported that *Ephedrae herba* (Mantani et al., 1999) and green-tea (Imanishi et al., 2002) extracts inhibit the growth of influenza virus with a similar mechanism of action.

Further experiments should be performed to clarify the exact mechanism of action of TTO that, as demonstrated for the macrolide antibiotics bafilomycin A1 and concanamycin A (Guinea and Carrasco, 1994; Natale and McCullough, 1998; Ochiai et al., 1995), could affect the vacuolar proton-ATPase activities.

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