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RESEARCH PAPER

Boosting Antioxidants by Lipophilization: A Strategy to Increase Cell Uptake and Target Mitochondria

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ABSTRACT

Purpose To explore the possibility to boost phenolic antioxidants through their structural modification by lipophilization and check the influence of such covalent modification on cellular uptake and mitochondria targeting.

Methods Rosmarinic acid was lipophilized by various aliphatic chain lengths (butyl, octyl, decyl, dodecyl, hexadecyl, and octadecyl) to give rosmarinate alkyl esters which were then evaluated for their ability (i) to reduce the level of reactive oxygen species (ROS) using 2′,7′-dichlorodihydrofluorescein diacetate probe, (ii) to cross fibroblast cell membranes using confocal microscopy, and (iii) to target mitochondria using MitoTracker® Red CMXRos.

Results Increasing the chain length led to an improvement of the antioxidant activity until a threshold is reached for medium chain (10 carbon atoms) and beyond which lengthening resulted in a decrease of activity. This nonlinear phenomenon—also known as the cut-off effect—is discussed here in connection to the previously similar results observed in emulsified, liposomal, and cellular systems. Moreover, butyl, octyl, and decyl rosmarinates passed through the membranes in less than 15 min, whereas longer esters did not cross membranes and formed extracellular aggregates. Besides cell uptake, alkyl chain length also determined the subcellular localization of esters: mitochondria for medium chains esters, cytosol for short chains and extracellular media for longer chains.

Conclusion The localization of antioxidants within mitochondria, the major site and target of ROS, conferred an advantage to medium chain rosmarinates compared to both short and long chains. In conjunction with changes in cellular uptake, this result may explain the observed decrease of antioxidant activity when lengthening the lipid chain of esters. This brings a proof-of-concept that grafting medium chain allows the design of mitochondriotropic antioxidants.

KEY WORDS antioxidants · cut-off effect · lipophilization · mitochondria · oxidative stress · polyphenols · rosmarinate esters

INTRODUCTION

Oxidative stress resulting from an imbalance between ROS production and antioxidant defenses may induce lipid, protein and DNA oxidative damage and therefore have important physiopathological consequences (1–5). Mitochondria, a major source of ROS (6–9), are involved in a variety of key cellular regulatory processes, including ATP production, intracellular Ca^{2+} regulation, and play an important role in fuel metabolism, cell proliferation, differentiation (10,11) and apoptosis (12,13). As these organelles are not only ROS producers at the cell level but also direct targets of these molecules able to alter their multiple activities, mitochondrial oxidative stress attracted a great interest in cancer research (14–16). Consequently, the development of antioxidant strategies able to prevent this oxidative stress through mitochondrial ROS scavenging is thus of particular interest (16–18).

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Among natural antioxidants, phenolic compounds are ubiquitous in plants and are significant components of a diet rich in plant products. As such, they may participate in the well-proven protective action of this type of diet against chronic diseases associated with oxidative stress (19) such as diabetes (20), cardiovascular diseases (21,22) and cancers (23). However, most of these molecules are hydrophilic and their poor affinity for phospholipid membranes limits their use for pharmaceutical purposes, especially in the design of drug-delivery systems (24).

To boost the antioxidant activity of phenolic compounds, Figueroa-Espinoza and Villeneuve (25) proposed to graft aliphatic chains onto phenolic compounds to improve their antioxidant properties along with making an amphiphile or a lipid-soluble molecule. Following this proposal, recent results on homologous series of chlorogenate and rosmarinate alkyl esters have emerged from different studies using lipid-based non-living systems and cells and have profoundly changed our understanding of the relationship between hydrophobicity and antioxidant activity (26). Specifically, we reported in emulsified (27–30) and liposomal systems (31), as well as in ROS-overexpressing human dermal fibroblasts (26), that antioxidant activity of homologous series of phenolic acids increases as the alkyl chain is lengthened, with a threshold for a medium chain, beyond which further chain extension leads to a drastic decrease in this activity. This sudden drop of activity indicates that the relationship between antioxidant capacity and hydrophobicity of phenolics follows a nonlinear relation also known as the *cut-off* effect. Thus, the lipophilization technique appears to be a double-edge sword: if the grafted aliphatic chain is too short or too long, the resulting antioxidant activity will not be optimal (32). However, the question remains why a reduction of antioxidant activity occurs beyond a critical chain length. The search for mechanisms explaining the cut-off effect thus hold great promise for mastering the lipophilization technique and to design promising new antioxidants in a rational fashion.

Among polyphenols, rosmarinic acid displays the best antioxidant activity of all the hydroxycinnamic acids tested for the H-donation capacity of their phenolic hydroxyl groups (rosmarinic acid > chorogenic acid > caffeic acid > ferulic acid > coumaric acid) (19). Its two *o*-diphenol (catechol) structures lead to the best H-donation capacity for the corresponding hydroxyl groups and consequently to good antioxidant properties (33–35). In addition, rosmarinic acid is known for displaying a wide spectrum of therapeutical properties in diseases including cardiopathologies (36,37), diabetes (37), inflammation (38) or Alzheimer's disease (39,40). However, data showed that rosmarinic acid tends to mainly localize in cell membrane (41). Therefore, a scientific challenge arose to enhance the bioavailability of phenolic compounds in order to prevent oxidative stress-related diseases and, as much as possible, to target a specific subcellular site. In this work, the lipophilization of rosmarinic acid has been performed using esterification with varying alkyl chain lengths (methyl, butyl, octyl, decyl, dodecyl, hexadecyl, and octadecyl) (42). The antioxidant activity of this complete homologous series of rosmarinate "phenolipids" (phenol + lipid) was assessed in relation to their cellular uptake and localization, using a cell line spontaneously producing high ROS amounts (43).

MATERIALS AND METHODS

Chemicals

Rosmarinic acid, Dulbecco's modified Eagle's medium (DMEM, 4.5 g/l glucose) with and without red phenol, trypsin solution (2.5 mM), methanol, butanol, octanol, decanol, dodecanol, hexadecanol, and octadecanol were purchased from Sigma Aldrich (Saint-Quentin, France). Propidium iodide, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA (λ_{ex} : 485 nm/λ_{em} : 530 nm)), and MitoTracker® Red CMXRos (λ_{ex}: 543 nm/λ_{em}: 599 nm) were purchased from Invitrogen. Rosmarinate alkyl esters (Fig. 1) were synthesized by chemical esterification with Amberlyst IR 120H as described by Lecomte *et al.* (42) to obtain butyl, octyl, decyl, dodecyl, hexadecyl and octadecyl rosmarinates.

ROS Level Measurement

Antioxidant activity of rosmarinic acid and its alkyl esters with varying chain length (butyl, octyl, decyl, dodecyl, hexadecyl, and octadecyl) was measured using a fibroblast cell line genetically modified to spontaneously produce high ROS levels of mitochondrial origin (43). Antioxidant activity of these alkyl rosmarinates was evaluated at various concentrations (0.5, 2.5, 5, 10, 25, 50 μM) through their ability to inhibit H_2DCF-DA fluorescence. Black 96-well microplates with flat and transparent bottom (Greiner, Frickenhausen, Germany) were seeded at a cellular density of 10,500 cells/well. Cells were allowed to grow in DMEM (4.5 g/l glucose) supplemented with gentamicin (50 μg/ml), amphotericin B (50 μg/ml) and foetal calf serum (10%) at 37°C, 5% CO_2 and 100% humidity. After 24 h incubation with phenolic compounds, the medium was removed. Adherent cells were washed twice with 50 μl/well DMEM without red phenol. A measurement of the fluorescent background (λ_{ex} : 485 nm/λ_{em} : 530 nm) was performed using a microplate reader spectrofluorimeter (Synergy 2 BioTek, Biotek Instruments, Inc., Winooski, USA). Cells were incubated with 100 μl of a 10 μM solution of H_2DCF-DA prepared in DMEM without red phenol, for 20 min at 37°C in the dark. After removal of the H_2DCF-DA solution, each well was washed twice using 50 μl DMEM without red phenol and filled with 100 μl DMEM without red phenol. The microplate

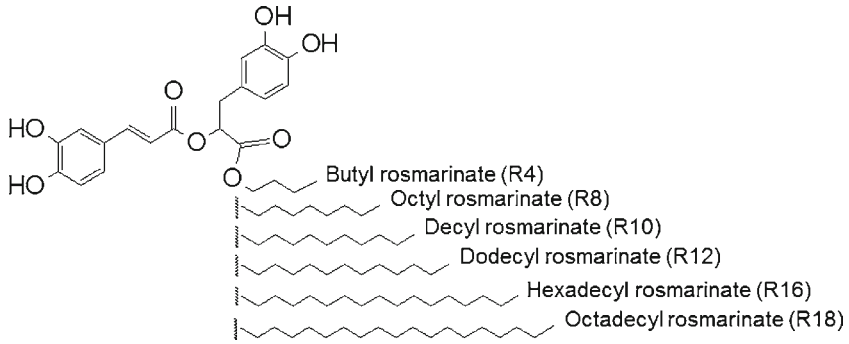

Fig. 1 Chemical structure of rosmarinate esters: Rosmarinic acid was esterified by different alkyl chain (R4: butyl, R8: octyl, R10: decyl, R12: dodecyl, R16: hexadecyl, R18: octadecyl) with Amberlyst IR 120H.

covered by an aluminum foil was then incubated for 5 min at 37°C prior to H_2DCF-DA fluorescence measurement (44). As described by Laguerre *et al.* (26), the ROS level was calculated as the percentage of fluorescence inhibition of H_2DCF-DA, in presence of the tested phenolic relative to the phenolic free control; this value was normalized relatively to DNA content.

For the kinetic study, after the incubation of H_2DCF-DA, each rosmarinate alkyl ester was incubated and the ROS level was determined every 5 min for 2 h.

Experiments were repeated three times ($n=3$) in three different microplates seeded from different cell passages (each microplate comprising already duplicate wells for each sample). Results are expressed as mean ± standard deviation.

Confocal Microscopy

Excitation and emission spectra of rosmarinic acid, decyl, and octadecyl rosmarinates were determined in phosphate buffer solution (PBS) at pH 7.2 (10 μM final concentration) in a 1-cm quartz cuvette. λmax for excitation and emission were found to be 395 and 450 nm, respectively. In confocal experiments (LSM 510 META, Zeiss), rosmarinic acid and its alkyl esters can be visualized by setting excitation and emission wavelengths at 405 nm and 420–480 nm, respectively. Image acquisition was obtained from 1-μm sections using the LSM browser software. To study the cellular uptake of rosmarinic acid and its alkyls esters, 25 μM of compounds were incubated with cells for 15, 35, and 120 min in DMEM with serum. Cells were washed with DMEM without phenol red to remove both DMEM and serum before image acquisition.

To study localization of decyl rosmarinate in mitochondria, cells were incubated for 2 h with both MitoTracker® Red CMXRos (10 μM) and decyl rosmarinate. Colocalization was assessed by confocal microscopy using two channels: 585–615 nm (Mitotracker in red) and 420–480 nm (rosmarinate in blue)

Critical Micelle Concentration (CMC) Determination of Decyl Rosmarinate

Ethanolic solutions of rosmarinate decyl (R10) were prepared at concentrations ranging from 0.5 to 20 mM and then diluted at 1/1,000 in PBS (pH 7.2) to reach concentrations between 0.5 and 20 μM. Surface tension measurements were performed at 37°C with dynamic drop tensiometer (Tracker TECLIS). Each surface tension measurement was done after 20 min to allow the system to reach equilibrium. The surface tension decreases when the concentration of decyl rosmarinate increases until a threshold is attained and beyond which adding rosmarinate does not impact the surface tension. The intersection of the two linear portions in the graph determines the CMC at which decyl rosmarinate starts to self-assemble in monodispersed micelles.

Hydrolytic Stability of Rosmarinate Alkyl Esters

Ethanolic stock solutions of 25 mM rosmarinate esters were diluted (1/1,000) in PBS (pH 7.2) and stored at 37°C for 24 h in the dark. Then, 25 μL of these solutions were analyzed using a Dionex Ultimate 3,000 HPLC system (Dionex, Jouy en Josas, France). Separation was carried out on an ACE C-18 column (5 μm, 250×4.6 mm, 100 Å) (AIT France, Houilles, France) at a flow rate of 1 mL/min, using 3 mM H_3PO_4 (solvent A) and pure methanol (solvent B). Elution was performed in a linear gradient from 95 to 25% A for 25 min and from 25 to 0% A for 15 min, then 0% A for 10 min, and finally back to initial conditions in 5 min. Rosmarinic acid and its alkyl esters were detected at 328 nm using an Ultimate 3,000 Variable Wavelength Detector and peak integration was performed using Chromeleon software (Version 6.8).

Cytotoxicity

The cytotoxicity of rosmarinic acid and its alkyl esters was assessed using propidium iodide fluorescence measured with a Gallios flow cytometer (Beckman Coulter). 3×10^6 cells were seeded into 6-well plates with 1 ml of culture medium. After 24 h, various concentrations of rosmarinic acid or its esters were added to the culture medium at (0.5, 2.5, 5, 10, 25, and 50 μM). After 24 h of incubation with rosmarinate esters, fibroblasts were harvested with trypsin, centrifuged, suspended in 800 μl of DMEM (without red phenol) containing 27 μg/ml of propidium iodide. Finally, samples were analyzed by flow

cytometry at 488 nm (excitation) and 585/42 nm (emission band-pass). Experiments were repeated three times ($n=3$) in three different plates seeded from different cell passages. Results are expressed as mean ± standard deviation.

Curve-Fitting

The CurveExpert 1.3 shareware (Copyright © 1995–2001 Daniel Hyams) was used in curve-fitting procedures using a quadratic formula for calculation of area under the concentration curves.

Statistical Analysis

Data are expressed as the mean ± standard deviation. The statistical significance of the differences between rosmarinic acid and its esters was analyzed with a two-way analysis of variance (ANOVA) and Mann-Whitney's test. Statistical significance was defined as $p<0.05$.

RESULTS

Antioxidant Activity After 24 h of Incubation

To overcome the difficulties inherent to the use of exogenous oxidant species, we have used a fibroblast cell line genetically modified to produce important amounts of oxidant species of mitochondrial origin (43). The influence of hydrophobicity on the antioxidant capacity of phenolic compounds was studied using a homologous series of alkyl esters of rosmarinic acid during 24 h of treatment. We found a dose-dependent inhibition of ROS levels for rosmarinic acid and all of its alkyl esters (Fig. 2). When using 0.5 µM of the rosmarinate alkyl esters, two groups appeared: the first one including rosmarinic acid (R0), butyl (R4), dodecyl (R12), hexadecyl (R16), and octadecyl (R18) rosmarinates did not display any antioxidant activity; the second including medium chain esters, octyl (R8) and decyl (R10) rosmarinates, causing a 33%-decrease of ROS levels. At 50 µM, three distinct groups were observed: i) polar compounds R0 and R4 exhibited a ROS level reduction of 42 and 35%, respectively; ii) R12, R16, and R18 had an intermediate antioxidant activity (respectively 77, 67 and 66%); iii) R8 and R10 medium chain rosmarinate esters had the highest reduction in ROS level (80 and 87% respectively).

Calculation of the area under the curve provided complementary data indicating that the order of antioxidant efficiency was: R0 (2.96)~R4 (2.87)~R16 (3.04)<R18 (3.51)<R12 (3.98)<<R8 (6.04)<R10 (7.67) (Fig. 3). From these results, it appears that the chain length exerts a nonlinear effect on antioxidant activity. Increasing the chain length leads to a rise in the antioxidant activity until a critical chain length reported

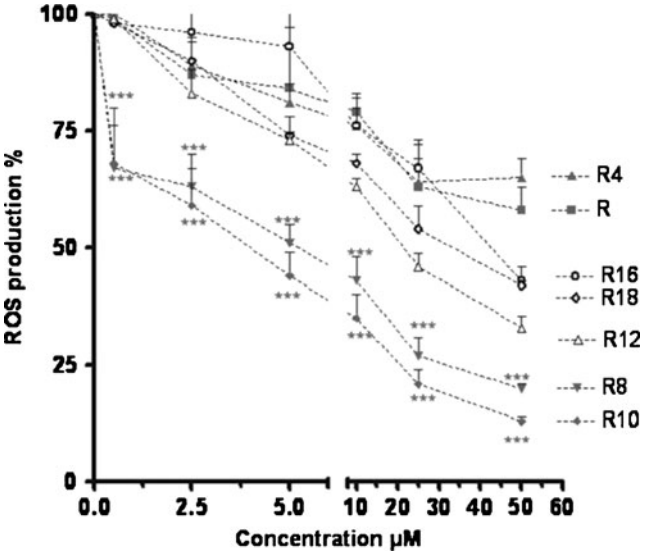

Fig. 2 Influence of a 24 h treatment with rosmarinic acid and its alkyl esters on ROS level in fibroblasts (% fluorescence H_2DCFDA relatively to control without rosmarinate esters): Cells were cultured in DMEM without red phenol supplemented with 10% (*v/v*) fetal calf serum, treated with 0, 0.5, 2.5, 5, 10, 25 and 50 µM phenolic compound or 0.1% (*v/v*) ethanol (control cells) during 24 h and incubated with 10 µM of H2DCFDA. ROS level was assessed using H_2DCF fluorescence using a microplate reader (λex: 485 nm/λem: 530 nm). Results are expressed as mean ± SD ($n=3$). *$p<0.05$ and *** $p<0.001$ (ANOVA $n=3$) when compared with rosmarinic acid (R0).

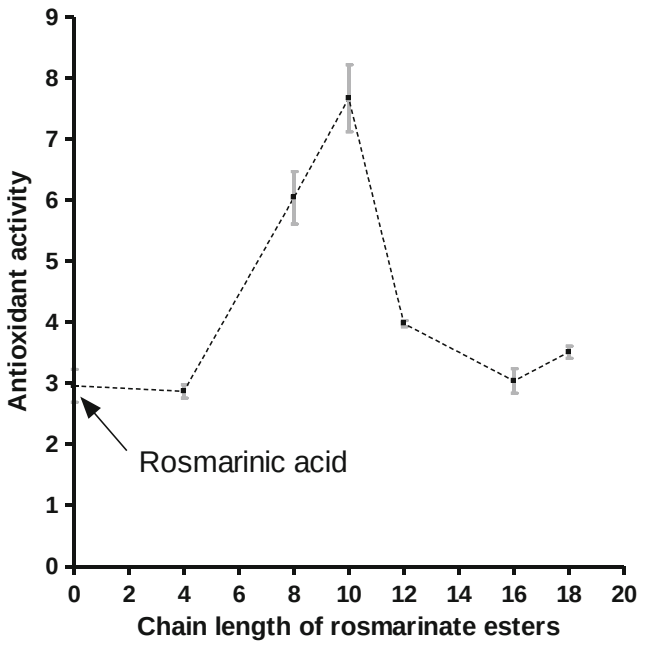

Fig. 3 Influence of the chain length of rosmarinic acid on its antioxidant activity after 24 h of treatment. Antioxidant activity was expressed as a function of the alkyl chain length, as 1/curve area of ROS level *versus* concentration (Fig. 2) × 10,000. Data are derived from three independent experiments. *$p<0.05$, **$p<0.01$ (student *t* test) when compared with rosmarinic acid (R0).

for octyl and decyl chains. Any additional lengthening beyond this threshold resulted in a strong decrease of this activity.

Kinetic Study of the Antioxidant Activity

To better understand how the alkyl chain influenced antioxidant activity, we kinetically studied the ability of each molecule to reduce ROS level. The sudden collapse of the antioxidant activity beyond a critical chain length observed after a 24 h treatment was also detected after 15 min. Indeed, long chain esters (R16 and R18) reduced ROS levels much more slowly than medium (R8, R10), short chain esters (R4) and rosmarinic acid (R0). As expected, this difference was exacerbated after 2 h when ROS levels in R0, R4, R8, and R10 treated cells was decreased to 50–60% of the ROS level measured in control cells, while it did not decrease below 90% of the control for R16 and R18 (Fig. 4). Intriguingly, similar reductions were recorded for R0, R4, R8 and R10 after 2 h, whereas after 24 h treatment R8 and R10 were much more effective than R0 and R4 (Figs. 2 and 3).

Cell Uptake and Localization

To identify the differences in the antioxidant efficiency of these rosmarinate alkyl esters, we studied their cellular internalization at 25 µM. Using fluorescence confocal microscopy, we found that cell entry differed according to the length of the alkyl chain. Whereas significant amounts of R4, R8 and R10 were detected inside fibroblasts as early as 15 min, the level of internalized rosmarinic acid (R0) was very low at this time point (Fig. 5). This may account for the observed differences in antioxidant activity recorded between R0 and medium chain rosmarinate esters (R8 and R10) from 30 to 50 min ($p<0.05$). In addition, long chain rosmarinate esters (R16 and R18) were essentially observed as extracellular aggregates. After 2 h of antioxidant presence, significant amounts of cellular rosmarinic acid and some of its alkyl esters were detected, but cell uptake of R16 and R18 was clearly slower when compared to others.

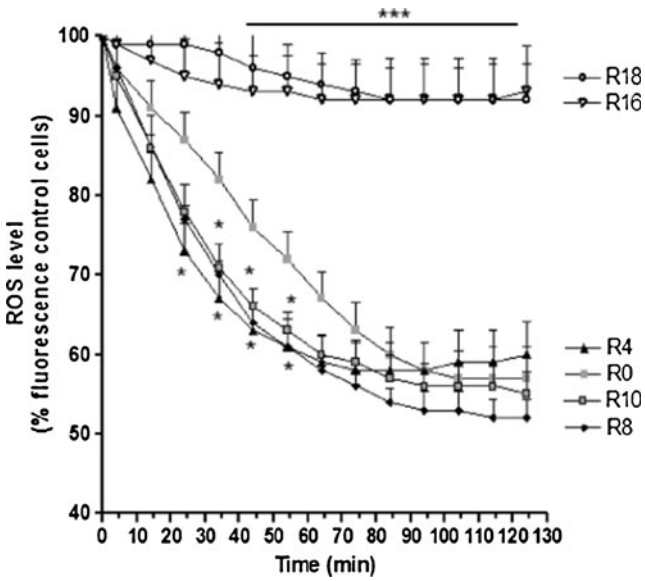

Fig. 4 Time related changes of ROS level during a 2 h treatment with rosmarinic acid and its alkyl esters (% fluorescence H_2DCFDA relatively to control cells without rosmarinate esters). Cells were cultured in DMEM without red phenol supplemented with 10% (*v/v*) fetal calf serum, treated with 25 µM phenolic compound or 0.1% (*v/v*) ethanol (control cells) during 2 h and incubated with 10 µM of H_2DCFDA. ROS level was assessed using H_2DCF fluorescence using a microplate reader (λex: 485 nm/λem: 530 nm). Results are expressed as mean ± SD ($n=3$). *$p<0.05$ and ***$p<0.001$ (student *t* test) when compared with rosmarinic acid (R0).

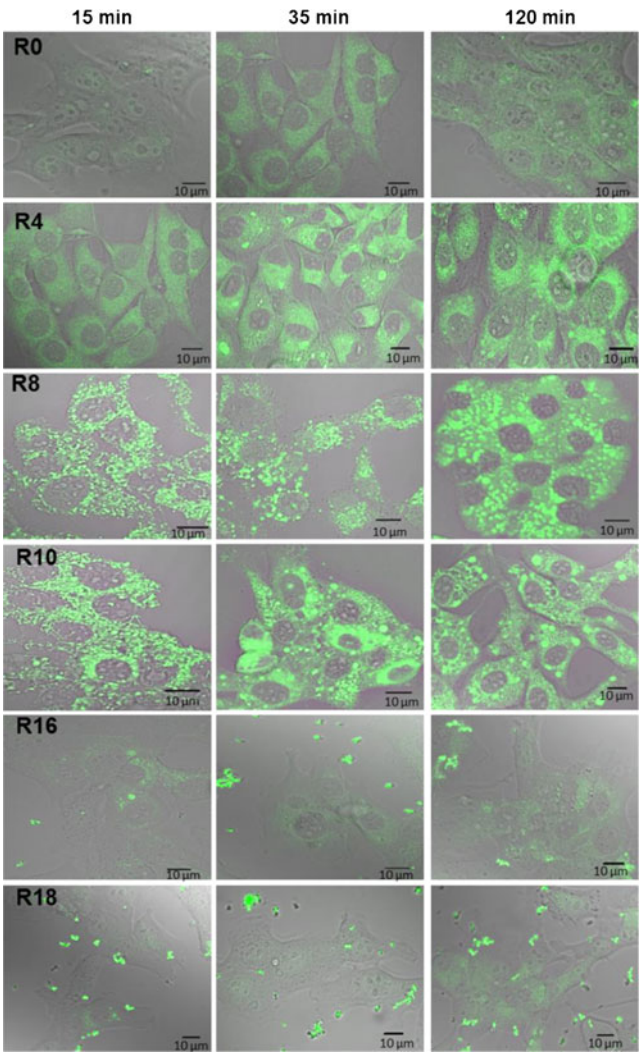

Fig. 5 Cellular localization of rosmarinic acid and its esters: Cells were cultured in DMEM without red phenol supplemented with 10% (*v/v*) fetal calf serum, treated with 25 µM phenolic compound or 0.1% (*v/v*) ethanol (control cells) during 15 min, 35 min and 2 h. The fluorescence of rosmarinic acid and its alkyl esters was monitored using confocal microscopy (λex: 405 nm, λem: 420–480 nm), magnitude x630. These microphotographs are representative of 3 independent experiments.

Furthermore, in contrast to R0 and R4 whose distribution within cytosol is diffuse, R8 and R10 presented a clear punctiform pattern suggesting a possible mitochondrial localization.

Subcellular Localization

In order to test the possibility that medium chain rosmarinate alkyl esters could be localized in mitochondria, we labeled these organelles with MitoTracker. We first established that addition in a test tube of all rosmarinate alkyl esters as well as free rosmarinic acid in a MitoTracker solution in PBS induced an immediate collapse of the fluorescence of these compounds (Fig. 6), as already shown for adaphostin with the MitoTracker CMXRos probes (45). Consequently, the presence of rosmarinate alkyl esters in mitochondria should result in a similar disappearance of MitoTracker fluorescence. After 2 h incubation of fibroblasts with MitoTracker and 25 μM phenolics (R0, R4, R10, and R18), R10 specifically induced an efficient MitoTracker fluorescence inhibition, in contrast to R0, R4 and R18 (Fig. 7), thus demonstrating colocalization in the organelles of MitoTracker and R10, but not with R0, R4 and R18. MitoTracker fluorescence inhibition induced by R10 was also recorded after a 24 h treatment (data not shown). Overall, these results established that the higher antioxidant activity of R10 at 24 h could be also explained by its ability to target mitochondria, a major site of ROS production.

Cytotoxicity Induced by Rosmarinate Alkyl Esters

After 2 or 24 h of rosmarinate esters treatment, R10 in contrast to R0, R4 and R18, was detected as round shaped vacuoles or aggregates suggesting that R10 could induce cellular damages (Figs. 5 and 7). This observation well agrees with the CMC measured for R10 (8.6 μM) by tensiometry (Fig. 8), suggesting that at the concentration used in fibroblasts (25 μM), R10 could induce intracellular micelle or aggregate formation after efficiently crossing the cell membrane. This result led us to assess possible rosmarinate alkyl esters cytotoxicity using propidium iodide incorporation. After 24 h of treatment with 50 μM phenolipids, R8 and R10 exerted the highest cytotoxic activity ($P<0.001$), when compared to other lipophilized rosmarinates (Fig. 9). Cell viability ranged in the following order: R0 (96%)>R18 (84%)~R4 (83%)~R16 (78%)>R8 (50%)>R10 (15%). Interestingly, the range of cytotoxicity positively correlated with the antioxidant activity and followed the same nonlinear trend relatively to the alkyl chain length.

DISCUSSION

Although hydrophobicity of antioxidant molecules is generally regarded as advantageous, the present study was initially aimed at answering the question of whether lengthening the alkyl chain necessarily leads to more efficient antioxidant

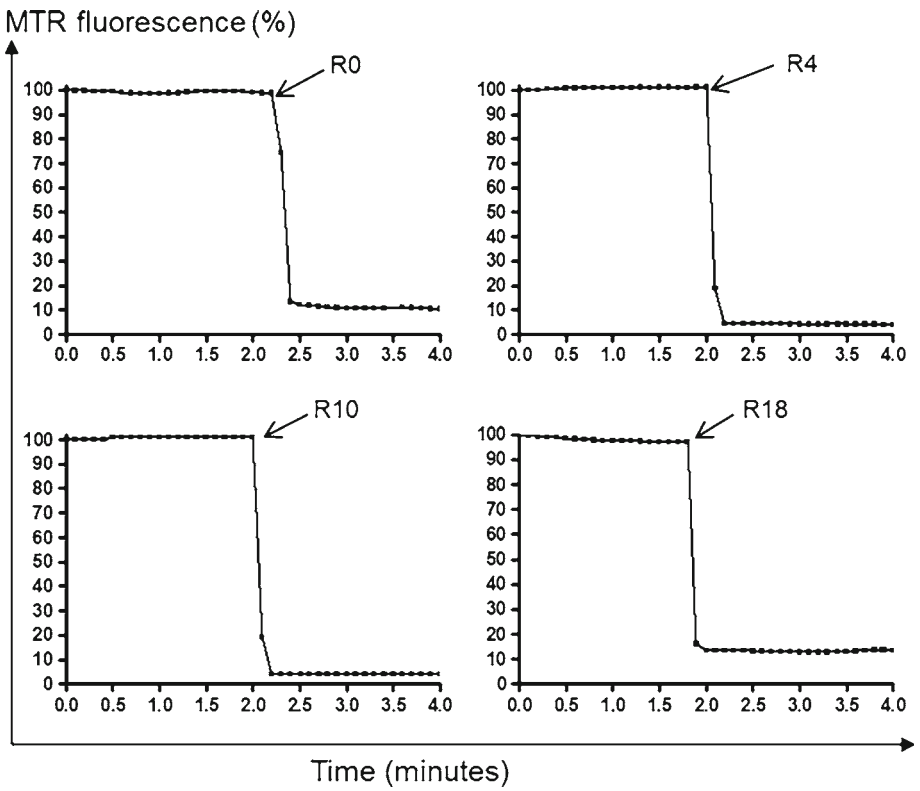

Fig. 6 Quenching of mitotracker (MTR) fluorescence by rosmarinate esters assessed by spectrophotometry. Mitotracker and rosmarinate esters (R0, R4, R10, R18) in equimolar amounts were solubilized in PBS (pH = 7.2) in a quartz cuve. Mitotracker fluorescence was monitored at a wavelength 590 nm and after five minutes, rosmarinate esters were directly added in the spectrophometer cuve (10 μM). Data are expressed as % of initial MTR fluorescence ($n = 3$).

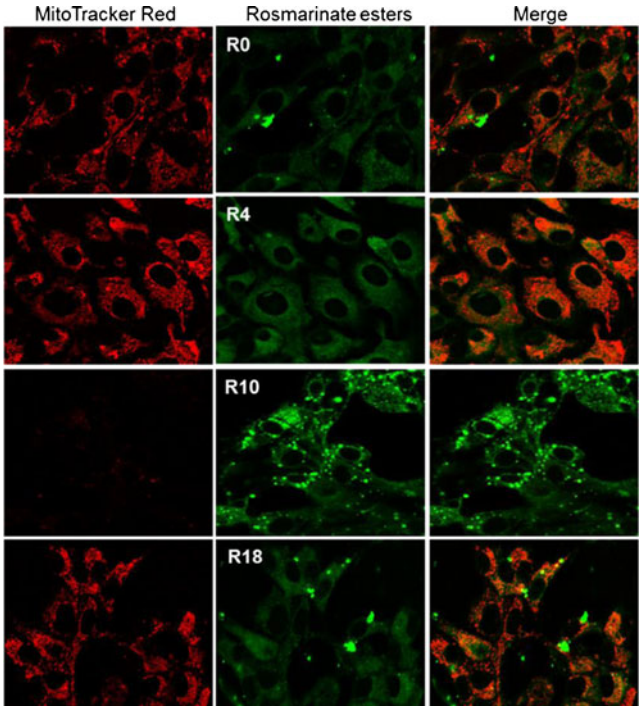

Fig. 7 Cellular localization of Mitotracker red and rosmarinic acid esters: Cells were cultured in DMEM without red phenol supplemented with 10% (*v/v*) fetal calf serum, treated with 25 μM phenolic compound or 0.1% (*v/v*) ethanol (control cells) during 2 h. The fluorescence of rosmarinic esters was monitored using confocal microscopy (λex: 405 nm, λem 420–480 nm). Fluorescence of MTR was monitored with the following wavelengths: λex: 543 nm; λem: 585–615 nm. Magnitudes x630, microphotographs are representative of 3 independent experiments.

activity. The salient result of this work is that beyond 8–10 carbon atoms, the chain lengthening alters the antioxidant activity of rosmarinate esters. In addition, this antioxidant

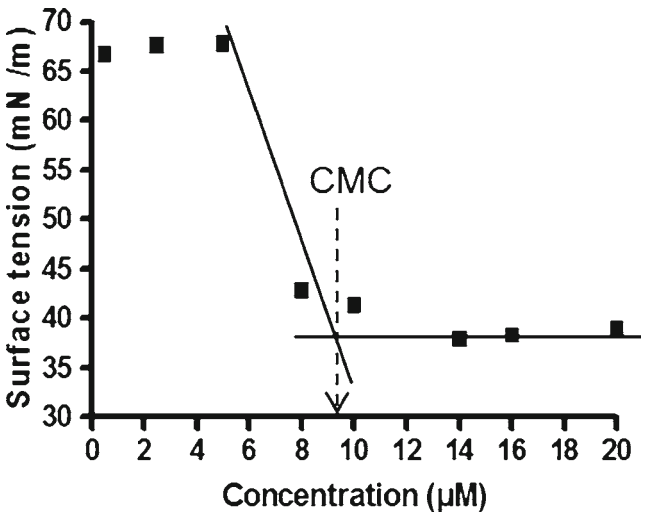

Fig. 8 Surface tension *versus* concentration plots of decyl rosmarinate. Surface tension was measured with a drop tensiometer (Tracker TECLIS). The intersection of the two linear portions of the curve determines the CMC.

activity of rosmarinate alkyl esters depends on their ability to cross the cell membrane and possibly on their subcellular localization. Even though hydrophobic compounds are able to cross phospholipid layers easily thanks to their membrane-like feature (fusogenicity), recent results show that highly lipophilic compounds may exhibit very poor antioxidant activity. This has been established when chain lengthening was performed in fibroblasts cell line for chlorogenate alkyl esters (26), in L6 rat myoblasts for hydroxytyrosyl alkyl esters (46) and fatty esters of hydroxytyrosol analogues (47). This nonlinear trend can be referred as a cut-off phenomenon.

In this study, we found that rosmarinate esters display different short (2 h) and long term (24 h) antioxidant activities, with respect to their chain length. During the two first hours of treatment, R4, R8, R10, and to a lesser extent, R0 exerted an efficient antioxidant activity. In contrast, R16 and R18 were totally inefficient during this early period. These differences fit well with our data concerning rosmarinate alkyl esters cell uptake. R4, R8, and R10 are easily detected inside cells as early as 15 min after rosmarinate alkyl esters addition in the culture medium and R16 and R18 cell uptake is slower than that observed for all other esters over 2 h. In addition, R0 cellular amounts remain below the detection threshold at this time. This could be partly explained by the observation that at physiological pH (pH = 7.2), the COOH group of R0 is deprotonated into COO^- group (pKa~4.5) thus hindering the crossing of R0 through membranes (24). However, after 2 h of phenolic compounds presence, despite detection of lower cellular amounts of R0 than R8 or R10 amounts, rosmarinic acid is, at this time, as efficient as R8 or R10. This result suggests that moderate amounts of rosmarinate esters are sufficient to scavenge the major part of cytosolic ROS. Clearly, the short term influence of phenolic compounds essentially depends on their ability to cross cell membranes. Consequently, the cut-off effect observed when using long chain esters partly relies on their poor ability to cross cell membranes.

The lipophilization strategy we used and the subsequent biological effect of the phenolipids relies on the strength of the ester bond formed between the alkyl chain and the rosmarinic acid moieties. Whether or not this ester bond is breakable in the tested conditions is an important question to address. In PBS at pH 7.2, we verified that the structure of all rosmarinate alkyl esters was preserved. HPLC analyses have indeed shown the presence of only one peak corresponding to the ester (for all compounds), even after 24 h of incubation with a pH ranging from 2 to 7.2 (data not shown). Consequently, this important stability in the culture medium led us to conclude that the ability of the different phenolipids to enter inside the cell, a major determinant of their antioxidant activity, depends on the length of the grafted alkyl chain. Moreover, once internalized into fibroblasts, the fact that rosmarinate alkyl esters partition quite differently as a function of the alkyl chain length after a very short period (less than 2 min, data not

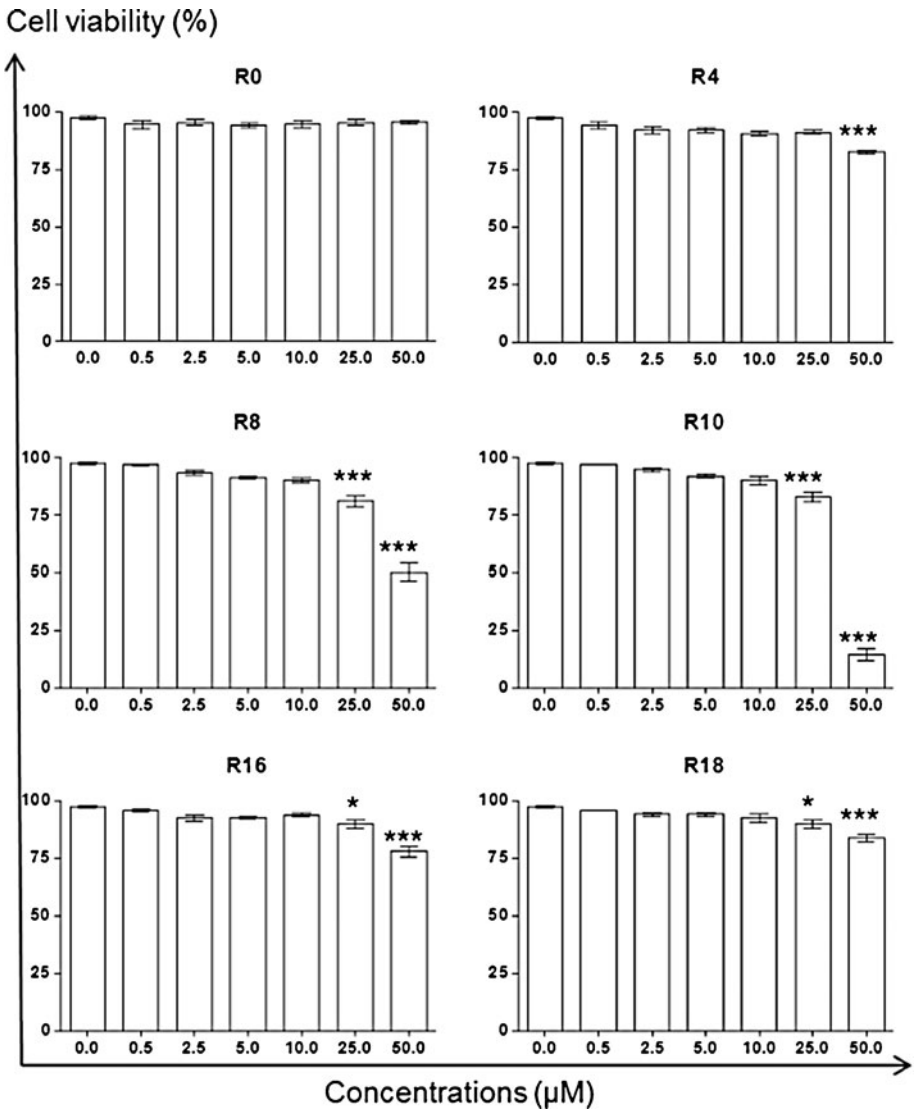

Fig. 9 Twenty four hours cytotoxicity of rosmarinate esters: Cells were cultured with DMEM supplemented with 10% (*v/v*) fetal calf serum, treated with 0 (control cells), 0.5, 2.5, 5, 10, 25 and 50 μM phenolic compound during 24 h. Cytotoxicity was assessed using propidium iodide and flow cytometry. Samples were analyzed at λex: 488 nm and λem: 585/42 nm. Results are expressed as mean ± standard deviation. $*p < 0.05$ and $***p < 0.001$ (ANOVA $n = 3$) when compared with the control (without rosmarinate ester).

shown), strongly suggest that the chain length is by itself the determinant of the cellular localization of each compound. However, more in-depth investigations should be carried out in future work regarding the metabolic fate of each phenolipid and their long term hydrolytic stability in mitochondria and in the cytosol.

Surprisingly, we found that the long term antioxidant influence of rosmarinic acid and its alkyl esters is not in good correlation with their short term influence. After 24 h of phenolic compounds presence in the culture medium, R8 and R10 displayed a significantly higher antioxidant activity than R0, R4, R12, R16, and R18. Indeed, another important observation is the punctiform cellular distribution observed for medium chain rosmarinate esters. Several data demonstrate a mitochondrial localization of R8 and R10: i) in solution, all phenolics induced a fluorescent inhibition of MitoTracker; ii) the punctiform localization of R10 is associated with an efficient inhibition of MitoTracker fluorescence, a dye specifically located in mitochondria and currently used for their visualization [41]; iii) R0, R4, and R18 presence in the cytosol did not induce any MitoTracker fluorescence inhibition, thus demonstrating that such fluorescence inhibition is not the result of rosmarinate alkyl esters presence in the cytosol; iv) the punctiform localization of R10 specifically induced a rapid and specific disappearance of MitoTracker fluorescence, indicating storage in a common cell compartment.

Interestingly, this mitochondrial localization could give a huge advantage of medium chain rosmarinate esters over the other chain lengths tested. Consequently, by scavenging ROS inside the organelle, R8 and R10 could strongly reduce the amount of ROS available for release in the cytosol, where they are detected by the H_2DCF-DA probe, thus explaining their higher antioxidant activity occurring after a 24 h treatment. While the short term antioxidant influence essentially depends on scavenging cytosolic ROS, and consequently on phenolipid cell uptake independently of their specific subcellular location,

the long term influence depends on their effect on the rate of mitochondrial ROS accumulation.

Hitherto, the main way to design mitochondriotropic antioxidants was to graft a triphenylphosphonium (TPP^+) cation to an antioxidant. The resulting molecule is then able to cross the matrix, driven by the constant membrane potential (−180 mV) that the organelle maintains across its lipid bilayer (negative inside). The idea to use these cations as "electric locomotives" targeting non-charged compounds to mitochondria was put forward by Liberman *et al* (48). Murphy and coworkers initiated the practical realization of this idea (17,49,50) by synthesizing and testing several mitochondria-targeted antioxidants conjugated to a lipophilic alkyl-TPP^+ cation (51). This successful approach has been extended since then to plastoquinone (52,53), vitamin E (MitoE) (54), resveratrol (55), and quercetin (56,57). An alternative strategy based on the grafting of a quaternary ammonium (QUATS) was proposed by Sheu *et al* (58) with choline esters of gluthation and *N*-acetylcysteine. Another way to design mitochondriotropic antioxidants has been developed with the so-called SS-peptides whose sequence targets mitochondria (59,60).

It is however difficult to envision how this latter strategy can be applicable to natural phenolics. In this context, our work may constitute a third way to target these organelles and may expand the molecular therapeutic arsenal against pathologies associated with mitochondria dysfunction. Moreover, the two catechol groups of rosmarinic acid represent one of the most efficient molecular structures to donate H-atoms to ROS (61). Further works should be done to see whether the design of mitochondriotropic antioxidants by lipophilization can be applied to other molecules than rosmarinate alkyl esters.

Besides their mitochondrial location assessed by a permanent MitoTracker fluorescence inhibition, high amounts of medium chain length rosmarinate esters produce cytosolic aggregates, in agreement with their self-assembly properties as measured by tensiometry (CMC R10 = 8.6 μM). Consequently, we studied cell toxicity for these series of phenolipids. As expected, in accordance with the antioxidant cut-off effect, R8 and R10 induced 17% of cell mortality, from the concentration of 25 μM and increases drastically at 50 μM. Such a cytotoxic effect of rosmarinic acid derivatives may be due to the high reducing activity of the two catechol functions (19,62), and their subsequent transformation into *o*-quinone which are extremely reactive with whatever nucleophiles that are present in the cell (amino-acids, proteins, lipid oxidation products, etc.). Their propensity to self-assemble into aggregates may also be involved in the mechanism of their cytotoxicity. An explanation for this could be that as R10 efficiently crosses the membrane at an extracellular concentration of 25 μM, the intracellular concentration could attain the CMC (8.6 μM), thus causing the formation of aggregates leading to cell toxicity. Nevertheless, when using lower levels, R8 and R10 displayed the most efficient antioxidant activities among all rosmarinate alkyl esters, without considerably affecting cell viability.

CONCLUSION

In summary, lipophilization on the carboxylic group of rosmarinic acid does not affect the two catechol groups responsible for the intrinsic antioxidant properties (reducing potential). In contrast, lipophilization influences the polarity of rosmarinic acid and, in doing so, induces important changes in its cell uptake and subcellular localization. As a result, *in cellulo* antioxidant activity was improved with increasing chain length until reaching a critical length (R10) beyond which antioxidant activity decreased. Our data explain satisfactorily the previously described cut-off effect obtained in cellular systems. Interestingly, medium chain rosmarinate esters are localized into mitochondria where they could not only act as scavengers of cytosolic ROS, but also inhibit mitochondrial ROS release by a classical ROS-scavenging process and/or by a possible inhibition of the respiratory chain. This could contribute to significantly protect the organelle itself against its adverse effects. Although we have observed that high levels of these phenolipids could affect cell viability, lower amounts exert an efficient antioxidant activity without such adverse consequences. The chief significance of this work lays in introducing the control of mitochondrial accumulation of antioxidants depending on the length of the grafted alkyl moiety, which could open up a novel way to design mitochondria-targeted antioxidants. Through lipophilization with medium chain, we pave the way for a rational strategy to improve cell antioxidant activity of natural vegetal compounds or synthetic drugs thus setting up a promising therapeutic approach for metabolic and degenerative diseases or cancers associated to an oxidative stress.

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