

Protective effects of the lipophilic redox conjugate tocopheryl succinyl-ethyl ferulate on HIV replication

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Abstract Previously, we demonstrated that ferulate ethyl and tocopherol reduced HIV replication. In this study, we investigate whether the conjugation of both compounds (*O*-tocopheryl succinyl *O*-ethyl ferulate) can increase HIV inhibition. We show here for the first time that *O*-tocopheryl succinyl *O*-ethyl ferulate inhibits 80% of HIV replication (HIV-1 acute infection and HIV transmission), inhibits cell lipoperoxidation and prevents cellular glutathione consumption. Compared to ferulate ethyl and tocopheryl succinyl, *O*-tocopheryl succinyl *O*-ethyl ferulate inhibits more HIV replication. This may be due in part to the great increase in the lipophilicity of this compound.

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Key words: Human immunodeficiency virus; Oxidative stress; Phenolic compound; Vitamin E; Lipophilic drug

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is the primary etiology of the acquired immunodeficiency syndrome (AIDS) [1–3]. In recent reports, cellular antioxidant status was suggested to be an important factor determining HIV activation [4]. It has been suggested that the shift in the antioxidant balance towards the pro-oxidant side contributes to the self-perpetuation of viral replication, immunodysregulation, and AIDS-Kaposi's sarcoma pathogenesis [4–8].

Already in the early stages of the disease, low plasma levels of acid-soluble thiol and reduced GSH are observed, and these continue to decline as the disease progresses [4,6]. Deficiencies in antioxidant enzymes (superoxide dismutase (SOD) [9], glutathione peroxidase and other antioxidants (ascorbic acid, tocopherol, carotenoids and selenium)) are observed in HIV-1 infected patients (reviewed in [4,10]). In addition, elevated serum levels of lipid peroxidation products, such as hydroperoxides and malondialdehyde (MDA), have been reported by several authors [4].

HIV-1 expression can be induced by oxidants such as hydrogen peroxide and singlet oxygen in vitro [11]. At the same time, HIV-1 can increase reactive oxygen species (ROS) accumulation via a *trans*-acting transcriptional activator (Tat) [12]. Tat acts as a pro-oxidant protein by activating proinflammatory cytokine secretion (TNF α , interleukin (IL)-1, IL6,

TGF β) [12] known to increase ROS production [13,14], and weakening cellular antioxidant defense mechanisms via suppression of Mn-SOD expression [15,16]. All these findings suggest that ROS and HIV infection form an autocatalytic vicious circle which finally renders cells more sensitive to the cytotoxic effects of TNF α and increases their susceptibility to undergo apoptosis [4,5,17–19].

Previously, we demonstrated that ferulic acid and tocopherol hemisuccinate reduced HIV replication in the chronically HIV-1 infected promonocytic U1 cell line [20,21]. In addition, we showed that ethyl ferulate, which is more lipophilic than ferulic acid, potentiated HIV inhibition [20]. In this study, we made a conjugate from the two molecules and investigated whether this conjugate, tocopheryl hemisuccinate with ethyl ferulate, can increase HIV inhibition.

2. Materials and methods

2.1. Chemicals

Ferulic acid and α -tocopherol acid succinate were purchased from Sigma. The structures of *O*-tocopheryl succinyl *O*-ethyl ferulate is presented in Fig. 1. It was synthesized as described in [22]. The physico-chemical study of this compound has been described by Barthelemy et al. [22].

2.2. Cells and culture conditions

The p24 antigen (HIV core protein) assay was chosen for this. Antigen levels were quantified by enzyme linked immunosorbent (ELISA, Du Pont Co., Delaware) in centrifuged culture supernatants from three different models of HIV infected cells:

2.2.1. U1 cell line. The U1 cell line was derived from chronically HIV-1 infected promonocytic U937 cells surviving an acute infection [23]. The cells were cultivated in a humidified 5% CO₂ environment at 37°C, in RPMI 1640 (Gibco BRL, New York) supplemented with 10% fetal calf serum, L-glutamine and penicillin-streptomycin (Gibco BRL). U1 cells (3×10^5 IU cells) were pretreated with or without *O*-tocopheryl succinyl *O*-ethyl ferulate for 1 h. Subsequently, 100 U/ml of recombinant TNF α (R&D Systems, Minneapolis, MN) was applied to each well. After incubation for 24 or 48 h, the culture medium was collected and assayed for p24 gag antigen levels. All the experiments were performed in triplicate. Cell survival was determined by trypan blue dye exclusion.

2.2.2. HIV propagation in peripheral blood mononuclear cells (PBMCs) activated with PHA and IL2. PBMCs obtained from HIV-1 seronegative donors were isolated by Ficoll-Hypaque density sedimentation (Pharmacia, Sweden) and incubated at a density of

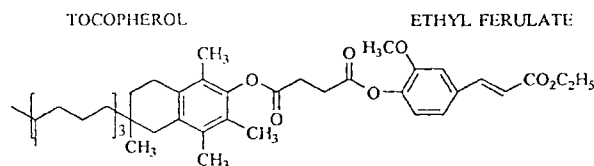


Fig. 1. Structures of *O*-tocopheryl succinyl *O*-ethyl ferulate.

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Abbreviations: HIV, human immunodeficiency virus; PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cell; TNF α , tumor necrosis factor alpha; ROS, reactive oxygen species; MDM, monocyte-derived macrophages

2×10^6 cells/ml in RPMI 1640, supplemented with 10% inactivated fetal calf serum, 3 mg/ml PHA (Sigma, Paris) and 5% v/v IL-2 (Gibco). After 48 h, the cells were washed and cocultivated with an equal number of PBMCs obtained from HIV-1 infected patients under the same culture conditions at a total cell concentration of 2×10^6 cells/ml in complete medium and incubated with or without *O*-tocopheryl succinyl *O*-ethyl ferulate. The supernatants of cocultures were assayed for p24 gag antigen in comparison to untreated cocultures after various cultivation periods (5, 7, 10 and 13 days).

2.2.3. HIV infection of monocyte-derived macrophages (MDM). Monocytes were isolated from healthy PBMCs by counter-current centrifuge elutriation (Beckman Instruments, California). Monocytes were cultured for 5 days in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum, L-glutamine and penicillin-streptomycin. This procedure permits monocyte differentiation and produces a maximal sensitivity for productive infection. The presence of the membrane antigens CD64, CD4, CD11B and MAXI was investigated by flow cytometry. After 5 days, 2×10^6 cells were washed in phosphate buffered saline (PBS) and infected for 3 h with HIV-1 Ba-L (10 TCID₅₀/10⁶ cells), kindly provided by Dr. A.M. Aubertin (Strasbourg, France). The medium was then removed and replaced twice weekly with fresh complete medium. After incubation for 7 and 14 days the culture medium was collected and assayed for p24 gag antigen levels. *O*-Tocopheryl succinyl *O*-ethyl ferulate was added both during the pre-infection period (24 h) and during the post-infection periods.

2.3. Measurement of lipid peroxidation

U1 cells (1×10^6 cells) were cultured in complete medium with *O*-Tocopheryl succinyl *O*-ethyl ferulate for 24 h. Subsequently, 100 U/ml of recombinant TNF α was added. After 24 h, cells were washed and pelleted and lipid peroxidation and GSH were measured. All experiments were performed in triplicate. Lipid peroxidation was evaluated by determining the U1 cell content of the thiobarbituric reactive substances (TBARS), according to the method of Yagi [24]. The relative fluorescence intensity of the TBARS was measured in a Kontron SFM 25 spectrofluorimeter with excitation and emission wavelengths of 515 and 548 nm, respectively. The concentration of lipid peroxides was expressed in terms of TBARS (nmol/mg protein), using MDA as a standard.

2.4. Determination of total glutathione

The total GSH level of U1 cells was determined by a kinetic assay involving the continuous reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) by NADPH as originally described by Tietze [25]. The glutathione results are expressed in nmol/mg protein.

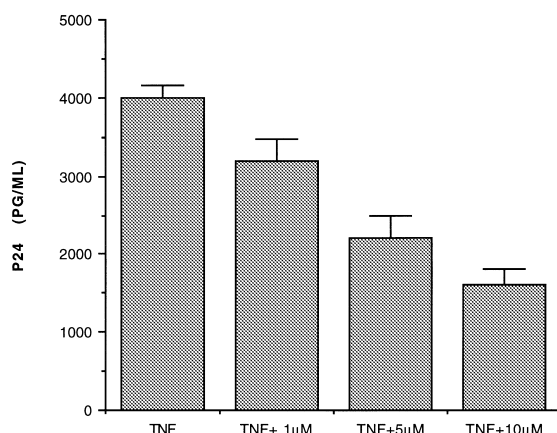


Fig. 2. Effect of *O*-tocopheryl succinyl *O*-ethyl ferulate on TNF α induced latency reactivation in U1 cells. Cells were pretreated with 1, 5 and 10 μ M of *O*-tocopheryl succinyl *O*-ethyl ferulate for 1 h and then stimulated with TNF α (100 U/ml). After 48 h, the supernatants were harvested and antigen p24 was measured. Values are mean \pm S.E., $n = 3$. * $P < 0.05$.

2.5. Reduction of DPPH

The scavenger potency of *O*-tocopheryl succinyl *O*-ethyl ferulate, ferulate ethyl and tocopherol was determined by the reduction of 1,1-diphenyl-2-picrylhydrazyl stable free radical. Equal volumes of equimolar solutions of DPPH and each test compound dissolved in ethanol were mixed. Ethanol was added to the control solution. The absorbance at 517 nm was measured after 20 min. The difference in absorbance between test compound and control was recorded as the reducing activity. Each experiment was performed in triplicate. The standard deviation of absorbance values was less than 10%.

2.6. Statistics

All results are expressed as mean \pm S.E. Comparisons of results were done using ANOVA test. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of *O*-tocopheryl succinyl *O*-ethyl ferulate on TNF α induced latency reactivation in U1 cells

In order to determine whether *O*-tocopheryl succinyl *O*-ethyl ferulate can modulate TNF α induced p24 antigen production, we pretreated the U1 cells with 1, 5, and 10 μ M of lipophilic redox conjugate for 1 h before TNF α stimulation. Antigen p24 was measured 48 h after TNF α stimulation. In the absence of stimulus, U1 cells produced very low amounts of p24 antigen [23]. Stimulation with TNF α resulted in the induction of p24 antigen in the culture supernatants (Fig. 2). Pretreatment for 1 h with *O*-tocopheryl succinyl *O*-ethyl ferulate induced a significant reduction of p24 antigen production, respectively 22, 41 and 63% 48 h after TNF α exposure (Fig. 2). In the absence of TNF α , *O*-tocopheryl succinyl *O*-ethyl ferulate had no effect on the viability of U1 cells (as determined by trypan blue dye exclusion) or on p24 antigen production (data not shown).

3.2. Effect of *O*-tocopheryl succinyl *O*-ethyl ferulate on HIV-1 propagation in PBMCs activated with PHA and IL2

PBMCs obtained from HIV-1 infected patients were incubated with PHA blasts prepared from HIV-1 seronegative controls in the presence or absence of *O*-tocopheryl succinyl *O*-ethyl ferulate. This compound was added at the start and at all time points of cell culture. Supernatants were harvested at days 7 and 14 and p24 antigen was quantified. In control coculture, we observed a progressive increase in supernatant p24 antigen expression (Table 1). 1 μ M of *O*-tocopheryl succinyl *O*-ethyl ferulate decreased p24 antigen expression (33% at day 14). This inhibitory effect increased with 5 μ M of *O*-

Table 1
Effects of *O*-tocopheryl succinyl *O*-ethyl ferulate on HIV-1 propagation in PBMCs activated with PHA and IL-2

Compound	Days of culture	
	7	14
0	150 \pm 13	15000 \pm 850
1 μ M	100 \pm 5*	10000 \pm 900*
5 μ M	90 \pm 7*	7500 \pm 400*
10 μ M	85 \pm 10*	3080 \pm 700*

PBMCs obtained from HIV-1 infected patients were incubated with PBMCs activated with PHA and IL-2 from HIV-1 seronegative controls in the presence of 0, 1, 5 and 10 μ M of *O*-tocopheryl succinyl *O*-ethyl ferulate. This compound was added at the start and at all time points of cell culture. Supernatants were harvested at days 7 and 14 and p24 antigen was quantified. Data shown are mean \pm S.E. of triplicate wells of p24 antigen (pg/ml), $n = 3$; * $P < 0.05$.

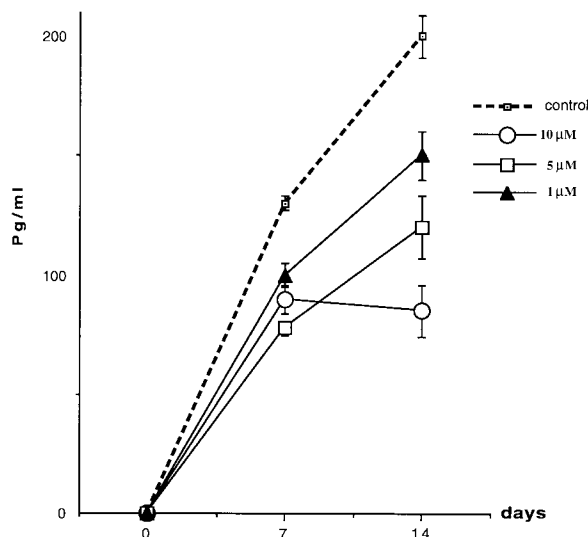


Fig. 3. Effects of *O*-tocopheryl succinyl *O*-ethyl ferulate in acute HIV infection of MDM. 1, 5 and 10 μ M of *O*-tocopheryl succinyl *O*-ethyl ferulate was added to medium 24 h before HIV infection (pre-infection period), at the time of HIV infection (day 0) and at days 3 and 7 (post-infection period). Supernatants were harvested and antigen p24 was measured at days 0, 7 and 14. Values are mean \pm S.E., $n=3$.

tocopheryl succinyl *O*-ethyl ferulate (50% at day 14), and at 10 μ M we obtained 80% of HIV inhibition. *O*-Tocopheryl succinyl *O*-ethyl ferulate at all concentrations used in these studies had no effect on viability of PBMCs, as determined by trypan blue dye exclusion (data not shown).

3.3. Effect of exogenous *O*-tocopheryl succinyl *O*-ethyl ferulate on HIV-1 infected macrophages

We studied the effects of *O*-tocopheryl succinyl *O*-ethyl ferulate in a model of acute HIV infection of primary differentiated monocytes. This compound was added to medium 24 h before HIV infection (pre-infection period), at the time of HIV infection (day 0) and at days 3 and 7 (post-infection period). *O*-Tocopheryl succinyl *O*-ethyl ferulate at 1, 5 and 10 μ M significantly reduced p24 antigen production respectively by 36, 45 and 52% at 7 days post-infection and by 40, 60 and 80% at 14 days post-infection (Fig. 3). No cytopathic effects were observed with *O*-tocopheryl succinyl *O*-ethyl ferulate treatment, using phase contrast microscopy.

3.4. Effect of *O*-tocopheryl succinyl *O*-ethyl ferulate on TNF α induced oxidative stress

Several lines of evidence implicate free radicals as a mediator of TNF induced cell injury characterized by a reduction in cellular GSH and increased accumulation of lipoperoxidation products [4,5]. In order to determine whether *O*-tocopheryl succinyl *O*-ethyl ferulate could protect U1 cells against TNF α ROS cytotoxic effects, we measured intracellular GSH and MDA production. TNF α induced a 56% decrease in cellular GSH content compared to control cells (45 ± 3 nmol/ 10^6 cells). In cells preloaded with *O*-tocopheryl succinyl *O*-ethyl ferulate (10 μ M), TNF α did not induce a decrease in the cells' GSH.

Concerning lipid peroxide concentration, a 120% increase in cellular TBARS was observed in TNF α treated cells com-

pared to control cells (2.0 ± 0.5 nmol/mg protein). *O*-Tocopheryl succinyl *O*-ethyl ferulate induced a marked decrease in TNF α induced TBARS production ($P < 0.004$). Culture supernatants did not contain any detectable amount of these lipoperoxidation products (data not shown).

3.5. Reducing activity against DPPH free radical

In order to determine the antioxidant activity of the conjugate and the two precursor molecules, we studied their reducing activities towards DPPH free radical. The percentage scavenging effect was calculated from the control where no compound was present. Ferulate exhibited higher scavenging activity, i.e. 64%. *O*-Tocopheryl succinyl *O*-ethyl ferulate and tocopherol hemisuccinate were completely 'inactive' because their chemical properties were modified during synthesis by blocking the phenolic groups.

4. Discussion

In the present study we show that pretreatment with the lipophilic redox conjugate *O*-tocopheryl succinyl *O*-ethyl ferulate significantly inhibited cell lipoperoxidation, prevented cellular GSH consumption and decreased HIV replication up to 80%.

Our results demonstrate that *O*-tocopheryl succinyl *O*-ethyl ferulate has an inhibitory effect on both HIV latency and development of HIV infection. To determine the effects of *O*-tocopheryl succinyl *O*-ethyl ferulate in a model system of viral latency, we used the U1 cell line, because it is a 'non-expressing cell', characterized by a state of relative latency and lack of detectable virion production [23]. HIV-1 expression can be induced in these cells by a variety of stimuli including inflammatory cytokines, oxidants [4] and clastogenic factors [19]. In this study, we used TNF α to activate HIV expression, because many of the symptoms associated with AIDS have been suggested to be TNF α -mediated [4–6,26]. TNF α is produced by macrophages and monocytes in response to mild oxidant stress and is found in excess in HIV/AIDS patients [4] which in turn is known to generate free radicals by a variety of cells [26]. TNF α can provide an 'amplification loop' which feeds back to further excite production of ROS from macrophages and neutrophils [4].

The effects of our lipophilic redox conjugate on acute HIV-1 infection were investigated in MDM infected with HIV-1 Ba-L. The reasons we selected the MDM and the macrophagotropic strain HIV-1 Ba-L in this model of HIV infection are that monocytes play a central role in the pathogenesis of HIV infection. The chronically infected monocyte serves as a constant reservoir of the virus [1]. Transmission of HIV-1 infection from one individual to another appears to be primarily due to monocytotropic strains that do not induce syncytia [27]. *O*-Tocopheryl succinyl *O*-ethyl ferulate inhibits HIV expression by 80%, indicating that in our experimental conditions, the lipophilic redox conjugate can interact at several steps of HIV expression.

To investigate the effect of *O*-tocopheryl succinyl *O*-ethyl ferulate in HIV transmission from HIV infected to uninfected PBMCs, we used PBMC coculture activated with PHA and IL2. *O*-Tocopheryl succinyl *O*-ethyl ferulate markedly decreased HIV expression and propagation in PBMC coculture. These findings strongly suggest the major involvement of oxidative stress in HIV expression. We can explain these results

by the findings of Kimura et al. who demonstrated the amplification of free radical production in cells of mononuclear phagocytes by HIV infection [28]. It was also shown that free radicals were able to enhance cell-to-cell transmission of HIV in a cultured human CD4 cell line [29].

We show here that when cells were preincubated with *O*-tocopheryl succinyl *O*-ethyl ferulate for 24 h, TNF induced MDA production was markedly inhibited and GSH decrease was prevented. Thus, we suggest that *O*-tocopheryl succinyl *O*-ethyl ferulate may increase the cell's ability to reduce the activation of the nuclear factor kappa-B (NF- κ B) and inhibit HIV replication.

Previously, we demonstrated that superoxide anion is implicated in chronic infection (U1 cell line), acute infection (HIV infected MDM) and in HIV propagation in PBMC coculture [17–19]. We also demonstrated that ferulic acid and its ester can inhibit HIV replication; this inhibition was more pronounced with ferulate ethyl than ferulic acid [20], which is more lipophilic as demonstrated by Barthelemy et al. [22]. In this study we show that conjugation of tocopherol acid succinate with ferulic acid ethyl significantly inhibits HIV replication in three different models of HIV infected cells. Compared to our previous studies [20,21], the HIV inhibition obtained with *O*-tocopheryl succinyl *O*-ethyl ferulate was higher (80%) than with ferulate ethyl and tocopherol. This may be explained by the fact that this conjugate has a higher lipophilicity score (than tocopheryl succinyl and ethyl ferulate) enabling a better incorporation into the lipidic part of the cell membrane and the exertion of its antioxidant effect at the true site of lipoperoxidation. This hypothesis is probable, because the conjugation of both compounds increases their lipophilicity scores [22]. In vitro, we found that *O*-tocopheryl succinyl *O*-ethyl ferulate was completely inactive towards DPPH free radical, because during chemical synthesis, all phenolic groups (OH) were blocked. However, further studies are being undertaken in our laboratory to investigate the antioxidant activity in vitro and whether this conjugate stays intact or can be separated into two moieties: tocopheryl hemisuccinate (remains in cell membrane) and ferulic acid (penetrates the cell).

Despite uncertainty about the specific intracellular mechanisms inhibited by *O*-tocopheryl succinyl *O*-ethyl ferulate, it remains from these ex vivo experiments that *O*-tocopheryl succinyl *O*-ethyl ferulate represents a non-toxic molecule with a high potentiality for therapeutic involvement against HIV-1 infection.

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