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ORIGINAL ARTICLE

In silico design, photostability and biological properties of the complex resveratrol/hydroxypropyl-β-cyclodextrin

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Abstract The topical administration of *trans*-resveratrol is limited because of its fast degradation upon UV irradiation. As assessed in the literature, cyclodextrins are able to protect the guest molecule against photodegradation. Accordingly, in this report the possibility of forming a stable complex between resveratrol and hydroxypropyl-β-cyclodextrin was firstly checked by docking strategies and then confirmed by solubility diagrams and differential scanning calorimetric measurements. The rate of degradation of resveratrol was then followed in different systems under UVA irradiation: the results showed the photoprotective effect of the complex. Moreover, the radical scavenging activity, the metal-chelating efficiency and the anti-lipoperoxidative potential of resveratrol were assessed: data showed that the inclusion phenomenon did not significatively interfere with these biological properties. Finally, in vitro experiments revealed that the skin accumulation of resveratrol was higher when released from the complex then when deposited alone.

Keywords Complex characterization · Docking · Lipoperoxidation · Metal-chelating · Photostability · Radical scavenging · Skin accumulation

Introduction

Resveratrol (*trans*-3, 5, 4′-trihydroxystilbene, RV) is a polyphenol phytoalexin naturally produced by several plants, mainly grapevine, as a metabolite in response to fungal infection or injury [1].

Together with other components of red wine like catechin, epicatechin, rutin and quercetin, *trans*-resveratrol has been shown to possess antioxidant properties and anti-atherogenic activity [2].

In few studies the antioxidant effect of resveratrol has been investigated in different lipid substrates like sunflower oil, rapeseed oil, margarine emulsion [3] and lard [4]. It was found that this molecule was rather effective against the oxidation of triacyglycerols even if it did not achieve the effectiveness of butylated hydroxytoluene (BHT) or of ascorbyl derivatives and that its antioxidant efficacy became more evident at later storage stages.

Recent studies have shown that resveratrol is also responsible for many other interesting biological effects, among them the scavenging of free radicals and the inhibition of the platelet aggregation. In addition it exhibits anti-inflammatory properties and cancer chemopreventive activity, derived from its ability to inhibit the cyclooxygenases and to induce apoptosis of neoplastic cells [5, 6].

In order to enlighten the radical-scavenging mechanism of *trans*-resveratrol, stationary γ-radiolytic experiments in liposomes and pulse radiolytic experiments in aqueous solutions were made. Applying the stationary γ-radiolysis together with the subsequent product analysis, reactions of lipid peroxyl radicals with *trans*-resveratrol and other natural antioxidants were investigated. It was found that *trans*-resveratrol was a better radical scavenger than vitamins E and C, but similar to the flavonoids epicatechin and quercetin [7].

Nowadays, resveratrol is under intensive study for its potential in cancer prevention [8]. In one study, it has been investigated whether resveratrol exerts anti-proliferative effects in human melanoma cells with different metastasic

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potential. Particularly, the effects of this polyphenol on the growth of weakly metastasic Line IV clone 3 and on autologous highly metastasic Line IV clone 1 cultured melanoma cells were examined. Comparable inhibition of growth and colony formation resulted from treatment by resveratrol in both cell lines [9].

Another paper reports that *trans*-resveratrol is adsorbed much more efficiently than (+)-catechin and quercetin in humans after oral consumption. Taking into account this result and the relative concentrations in red wine, *trans*-resveratrol was found to be the most effective anticancer polyphenol present in red wine [10].

Unfortunately, the use of *trans*-resveratrol in pharmaceutical and cosmetic field is problematic owing to its poor solubility in water and its fast degradation mainly linked to the isomerization from the active *trans* form to the inactive *cis* one [11]. Moreover it has been observed that an intense UV irradiation totally transforms resveratrol into a secondary compound, highly fluorescent in the UV range [12]. To date, the use of complexation with cyclodextrins was performed to decrease the *trans-cis* isomerization rate of resveratrol [13] and to dissolve this molecule before administration to rats [14] and to mice [15].

In this paper it was investigated whether the low water solubility and the fast degradation rate of *trans*-resveratrol could be overcome by the complexation with hydroxypropyl-β-cyclodextrin (HP-β-CD). In this regard, after a preliminary molecular modelling study to access the structural features of the complex, the kinetics of photodegradation of resveratrol, pure and complexed, were compared to evaluate the possible stabilizing ability of this cyclodextrin. The formation of the inclusion complex suggested by docking results was demonstrated by solubility diagrams and differential scanning calorimetry (DSC). Furthermore, the efficiency of resveratrol as antiradical, metal-chelating and inhibiting agent of lipoperoxidation was tested in the pure and complexed form to assess whether the inclusion in HP-β-CD could interfere with the activity of this molecule. Finally, the accumulation of *trans*-resveratrol in porcine ear skin was in vitro investigated.

Materials

Acetonitrile, absolute ethanol, butylated hydroxytoluene (BHT) and dichloromethane were from Carlo Erba (Rodano, Italy). Acetic acid, methanol, sodium azide, sodium dodecyl sulfate (SDS) were purchased from Fluka (Milan, Italy). Phosphoric acid (85%), 2-thiobarbituric acid (TBA), copper(II) chloride and 1-buthanol were from Merck (Milan, Italy). C12-15 alkylbenzoate (Finsolv®TN), hydroxyethyl cellulose (Natrosol®MR, HEC), octyl octanoate (Tegosoft® EE) were purchased from A.C.E.F (Fiorenzuola d'Arda, Italy). Polyacrylamide/laureth 7/C13-14 isoparaffin (Sepigel®305) arachidyl alcohol/behenyl alcohol/arachidyl glucoside (Montanov®202) and Caprylyl/Capryl glucoside (Oramix®CG110) were kind gifts from Seppic (Paris, France).

Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid), *trans*-resveratrol (*trans*-3, 5, 4′-trihydroxystilbene) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH˙) were from Sigma-Aldrich (Milan, Italy). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was a gift from La Roquette (Lestreme, France) while Phospholipon 90H was from Phospholipid (Cologne, Germany).

Apparatus

A Modulyo freeze dryer system (Edwards, West Sussex, UK) was used to prepare the inclusion complex of RV and HP-β-CD. The irradiation test was carried out in Pyrex® glass cells (5 mL volume) in a box equipped with an Actinic BLTLK40 W UVA lamp (Philips, Milan, Italy). HPLC analysis of RV was performed employing an apparatus (Shimadzu, Tokyo, Japan) consisting of a SPD-2A UV-Vis detector, a LC-6A pump unit control, a C-R3A chromatopac integrator and a RP-C18 column (80 × 4.6 mm; 5 µm. A lambda 2 UV-Vis spectrophotometer (Perkin Elmer, Waltham, MA) was also employed. The thermograms were obtained by a DSC-7 power compensation (Perkin Elmer, Waltham, MA). A DLS stirrer (Velp Scientifica, Milan, Italy), a SL-2 (Silverson, Bucks, England) and a T25 basic Ultra-Turrax® (IKA, Staufen, Germany) homogenizers were employed to prepare the samples. The measurements of pH were carried out with a microprocessor pHmeter (Hanna Instruments, Milan, Italy). A Rotavapor (Büchi, Flawil, Switzerland) was employed to prepare the liposomes.

Methods

Analysis of *trans*-resveratrol

A concentrated stock solution of RV in methanol was prepared to obtain the calibration curves. Diluted solutions over the range $2.5 \cdot 10^{-5}$–$8.5 \cdot 10^{-4}$ M were analyzed separately by UV-vis spectrophotometer and by HPLC. The molar extinction coefficient (ε) obtained spectrophotometrically at 306 nm was 36,200 M^{-1} cm^{-1} ($R^2 = 0.9975$).

For HPLC analysis a mixture of methanol/water/HCl (55/45/0.4) at a flow rate of 0.8 mL/min was used as mobile phase, the detection wavelength was 306 nm and the retention time was around 4.0 min. The equation of the obtained calibration curve was $y = 1.58 \cdot 10^7 x + 13{,}601$ ($R^2 = 0.9996$).

Inclusion complex and physical mixture preparation

Freeze drying technique was used to prepare the inclusion complex of RV and HP-β-CD. A mixture of RV and HP-β-CD (1:2 molar ratio) was prepared in water and shaken for 24 h in the dark. After equilibration the suspension was filtered and freeze dried before re-dissolution (0.5 mg) in 2.0 mL of methanol to assess the active loading by HPLC. The average percentage of active loaded was around 7.0% w/w with a percentage yield of 81.4%.

The physical mixture was prepared in the same molar ratio (1:2) by mixing appropriate amounts of solid components in a glass mortar.

Inclusion complex characterization

Molecular modelling

3D structures The HP-β-cyclodextrin structure was not found in the Cambridge Structural Database (CSD, version 5.29; data updates November 2007) and thus we built it by modifying with MOE [16] the methyl-β-cyclodextrin structure, in turn prepared as discussed in a previous paper [17]. This was possible since the two substituted CDs have similar degree of substitution (about 4 substituents per native CD molecule). Finally, the HP-β-CD geometry was minimized under MMFF94x and GB-SA conditions (RMS gradient <0.001 Å).

Resveratrol structure in its neutral form was simply downloaded from CSD (code DALGON) and checked with standard MOE tools.

Docking MOE-Dock methodology which consists of three steps (ligand conformational analysis, placement and scoring) was used [16]. For the initial systematic search, a random initial orientation was used, alpha triangle was the placement methodology and affinity dG scoring (a function that estimates the enthalpic contribution to the free energy of binding using a linear function) was the chosen scoring function.

One docking run was set up to generate 500 poses which were collected in a database. The resulting complexes were ranked by their scoring function (the lowest, the best). The best 50 poses out of 500 were minimised using GB-SA conditions (the newest version of the software automatically performs this operation), and ranked again by their scoring function.

Solubility diagram and stability constant

Phase solubility studies were performed according to Higuchi-Connors method [18]. An excess of RV (5 mg in 5 mL of water) was added to a series of vials containing increasing amounts of HP-β-CD in phosphate buffer at pH 6.0 and 8.0. The closed vials were shaken in the dark for 24 h at room temperature. After equilibration, each sample was centrifuged and analyzed by HPLC. The phase diagrams were constructed by plotting the total molar concentration of RV found in the solutions against the molar concentration of cyclodextrin. The stability constants (K_{st}) were calculated from the initial rising of the diagrams according the following equation:

$$K_{st} = \text{slope}/S_0(1 - \text{slope})$$

where the slope is obtained from the least squares linear regression of the molar concentration of RV versus the molar concentration of cyclodextrin and S_0 is the intrinsic solubility of resveratrol in the absence of cyclodextrin. The previous equation was applied by assuming a 1:1 stoichiometry for the inclusion complex.

DSC studies

The samples were placed in conventional aluminum pans and then heated under nitrogen flow at a scanning speed of 10 °C min^{-1} from 25 to 280 °C. The weight of each sample (pure RV, RV/HP-β-CD complex RV/HP-β-CD physical mixture and RV/HEC physical mixture) was such to have the same amount of RV in all of them.

Preparation of gel, gel emulsion and emulsion

HEC gel was prepared simply dispersing the opportune amount of HEC in water at 80 °C, mechanically stirring until room temperature was reached; aliquots of ethanol containing RV were then added.

The gel emulsion was prepared by adding to water opportune amounts of Sepigel 305 and Finsolv TN in which RV was previously dispersed, using an Ultra Turrax homogenizer.

The O/W emulsion was prepared by dispersing the melted lipid phase (Montanov 202, Tegosoft EE, RV) in water at 70 °C under homogenization. The emulsion was then cooled to room temperature under continuous stirring. The percentage compositions of HEC gel, gel emulsion and O/W emulsion are summarized in Table 1. These formulations were also prepared as described previously with RV/HP-β-CD by adding, in place of pure RV, appropriate amount of the complex to the aqueous phases.

Preparation of liposomes with RV

Lipid vesicles were prepared by a conventional rotary evaporation method. RV and Phospholipon 90H were dissolved in ethanol at the appropriate concentration and the solution obtained was then evaporated under vacuum by Rotavapor; solvent traces were removed under a flux of

Table 1 Percentage compositions of HEC gel, gel emulsion and O/W emulsion

Components	Gel (% w/w)	Gel emulsion (% w/w)	O/W emulsion (% w/w)
RV	0.005	0.005	0.005
HEC	2	–	–
Tegosoft EE	–	–	15
Finsolv TN	–	7	–
Montanov 202	–	–	6
Sepigel 305	–	2	–
Water	as to 100	as to 100	as to 100

nitrogen. The lipid film was then suspended in 10 mL water to be hydrated. The obtained suspension was magnetically stirred for 30 min and sonicated for few minutes.

To assess the entrapment efficiency the aqueous dispersion of liposomes was centrifuged at 24000 rpm, the supernatant was withdrawn and HPLC analyzed for RV determination.

Preparation of gel, gel emulsion and emulsion with RV-loaded liposomes

The gel, gel emulsion and emulsion were prepared as described above with 85% w/w of total water; the remaining water was substituted by the liposome dispersion containing the amount of RV necessary to obtain 0.2 mM (0.005% w/w) concentration.

Photostability studies

Experimental conditions

RV photodegradation experiments were performed using a BLTLK40 W UVA lamp (Philips) with a 320–400 nm range. An aliquots (10 g) of each system was introduced in Pyrex glass containers placed at 20 cm from the light source and maintained under continuous stirring. In such conditions the radiation power per surface unit was 2.9×10^{-4} W/cm^2. At scheduled times of 5 min over 1 h of total irradiation, fixed amounts of each sample were withdrawn and opportunely diluted for HPLC analysis.

Systems under study

- RV or RV/HP-β-CD in HEC gel without and with ethanol at increasing concentrations
- RV or RV/HP-β-CD in gel emulsion
- RV or RV/HP-β-CD in emulsion without and with BHT
- RV in liposome aqueous dispersion
- RV in liposomes dispersed in HEC gel
- RV in liposomes dispersed in gel emulsion
- RV in liposomes dispersed in emulsion

In all the systems studied RV was 0.005% w/w, except the liposome aqueous dispersion in which 0.05% w/w RV was contained.

Antiradical activity of RV

The radical scavenging activity of RV pure and included in HP-β-CD was determined by means of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$) assay. In its radical form, DPPH$^{\cdot}$ adsorbs at 515 nm but upon reduction by an antioxidant its adsorption decreases [19]. The reaction was started by the addition of 20 μL of RV or RV/HP-β-CD (0.5–10 μM final concentration) to 3.0 mL of DPPH$^{\cdot}$-saturated water/ethanol (90/10) mixture. 20 μL of Trolox 10 μM were also tested as reference. Absorbance was recorded after 10 min of magnetic stirring to reach the steady-state conditions.

Metal-chelating properties of RV

A solution of RV (10 μM) in water/acetonitrile (50/50, v/v) was spectrophotometrically analyzed at 306 nm in the absence (reference) and in the presence of increasing amounts of $CuCl_2$ (1.25, 2.50, 5.00, 10.00, 20.00 μM). The metal chelating efficiency of pure RV was compared to that of RV complexed with HP-β-CD at the same concentration.

In vitro skin accumulation studies

Ear skin from pigs was obtained freshly from a local slaughterhouse. Most of the underlying tissue was removed with a surgical scissor, making sure that the basal membrane was still present. The skin previously frozen at −18 °C was pre-equilibrated in 0.9% saline solution at 25 °C for 2 h before the experiments. Each circular piece of this skin was sandwiched between the two halves of a vertical Franz cell with the SC side facing the donor compartment. The donor phases (1 mL) consisted of:

- ethanol solution of RV (0.2 mM)
- gel emulsion containing RV/HP-β-CD (RV 0.2 mM)
- a mixture of 2% HEC gel and 2% Oramix CG110 containing RV/HP-β-CD (RV 0.2 mM)

The receiving compartments were filled with 0.9% w/w saline solution which was continuously stirred with a small magnetic bar. Each donor cell was sealed and covered with aluminum foil to prevent light exposure. At hourly intervals for 6 h, the receiving phases were taken, replaced with fresh SDS solution and HPLC analyzed. At the end of the

experiment (24 h) the receiving phase was analyzed while each skin piece was removed, washed with 50/50 water/methanol mixture, cut up and extracted with 3 mL of methanol. After 2 h of magnetic stirring, the suspensions were centrifuged and assayed by HPLC. The skin accumulation was expressed as μg of RV on cm^2 of skin diffusion area ($\mu g/cm^2$).

Assay of peroxidation in porcine skin

This assay is based upon the formation of a pink adduct (absorption maximum 532 nm) between TBA and malondialdehyde (MDA), a colorless end product of the skin lipid peroxide decomposition [20]. MDA was detected spectrophotometrically according to the TBA test described by Kovachich and Mishra [21] with the following modification. In one series of experiments ear porcine skin slices were cut in small pieces, transferred in Pyrex cells containing RV (0.2 mM) in 4% w/w SDS water solution and then irradiated for 3 h under UVA lamp under continuous stirring. An other series of ear porcine skin slices were irradiated in 4% w/w SDS solution containing the amount of RV/HP-β-CD necessary to obtain 0.2 mM concentration. The incubation medium used for reference slices was a 4% SDS solution free of RV. After irradiation the slices were dried under vacuum and then incubated for 24 h in 10 mL of dichloromethane to extract MDA. The organic solvent was evaporated under vacuum by Rotavapor; the residue was reconstituted with 3 mL of 8.1% w/w SDS aqueous solution and sonicated for 5 min. A 0.2 mL aliquot of each sample was added to 0.3 mL of distilled water, 1.5 mL of 1.0% w/w phosphoric acid and 1 mL of 0.6% w/w TBA. The mixture was heated for 45 min at 90–100 °C in water bath. After cooling in ice-bath to terminate the reaction, 1-butanol (4 mL) was added, centrifuged and the clear supernatant was spectrophotometrically analyzed to detect the TBA-MDA adduct. The final concentration of MDA generated during the reaction was calculated using a molar extinction coefficient of 7097 M^{-1} cm^{-1}. The same experiment was also performed in dark conditions by shielding all samples with aluminum foils.

Results and discussion

Molecular modelling

Docking is a computational strategy widely used in pharmaceutical and medicinal sciences that attempts to find the ''best'' matching between two molecules: a receptor and a ligand [22]. In a previous study we demonstrated that docking strategies could confirm experimental results about the inclusion complex of Trolox with methyl-β-cyclodextrin [17]. We thus reasoned that this time we could perform *in silico* simulations before setting up experimental measurements to avoid wasting time and resources in case of negative results.

In this study, we consider the HP-β-CD as the receptor and resveratrol as the ligand. As a comparison, the system Trolox /HP-β-CD was also investigated.

Numerical docking results suggest that resveratrol forms with HP-β-CD an inclusion complex more stable than that formed by Trolox. In fact, the best pose of the neutral resveratrol has a scoring function lower (=more stable) than the correspondent of Trolox (−19.52 and −14.98 kcal/mol, respectively). The difference of about 4 kcal/mol in scoring values is highly significant in this kind of studies to show a difference in affinity between two ligands and the same receptor.

Graphical docking results (Fig. 1 shows two cartoons of the best pose of resveratrol inside the HP-β-CD cavity) give some additional information. Firstly, the inclusion complex RV/HP-β-CD could have good photostability properties because of the poor exposition to the environment of the double bond which occupies an inner position inside the CD cavity (Fig. 1a). Secondly, the chelating properties of resveratrol could be conserved in the complex because the OH groups responsible for copper chelation are outside the cavity and thus easily accessible (Fig. 1a, red = oxygen atoms). Finally, docking results also suggest that the inclusion complex could be endowed with antioxidant properties. In fact, the visual inspection of Fig. 1b shows that all the three OH groups are easily accessible to radicals and this is in line with the paper of Stojanović et al. [7] that reports that all OH groups are responsible for radical-scavenging activity of *trans*-resveratrol, being more important the contribution of the *para*-hydroxyl group than that of the *meta*-hydroxyl groups.

Solubility diagrams and stability constants

A quantitative investigation of the inclusion complexation of RV in HP-β-CD was performed according to Higuchi and Connors method [18].

The solubility diagram of RV at increasing concentrations of HP-β-CD (at pH 6.0) is reported in Fig. 2, as example.

It was observed that the solubility of RV increased as the concentration of HP-β-CD was increased, displaying an A-type phase solubility diagram. The K_{st} was calculated from the initial linear portion of the curve, as early described and was 3189 M^{-1}. At pH 8.0 no significative deviation from this value was observed (data not shown). A study of RV complexes with β-CD, using RP-HPLC, is reported in the literature [23] indicating that 1:1 RV/CD

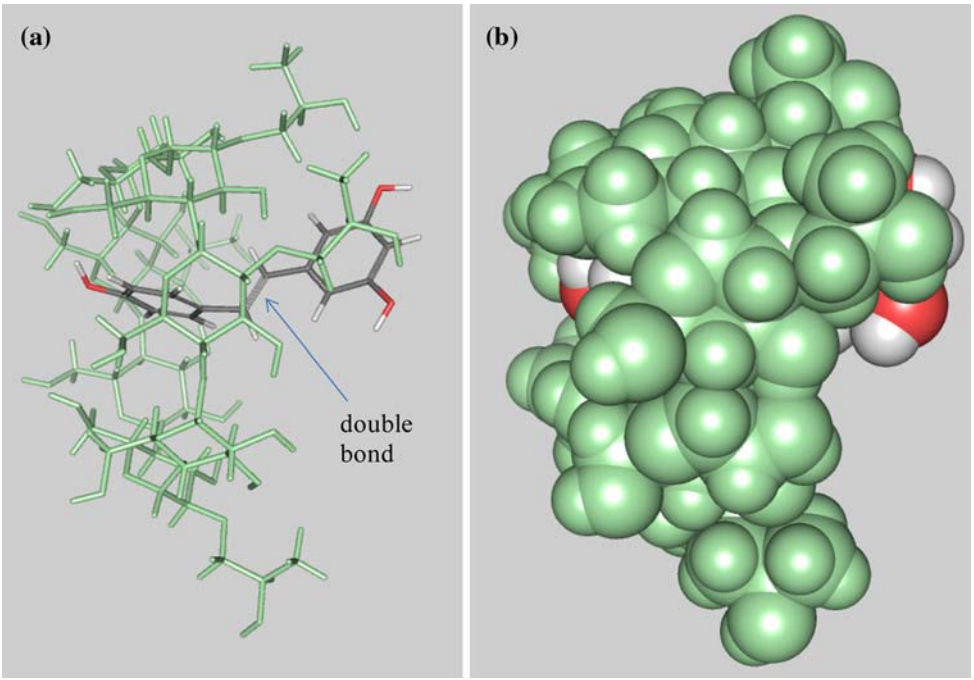

Fig. 1 Graphical docking results: **a** sticks model and **b** CPK visualization of the best pose of resveratrol obtained with docking simulation (HP-β-CD in *green*, resveratrol atoms: carbon = *dark grey*, oxygen = *red* and hydrogen = *white*)

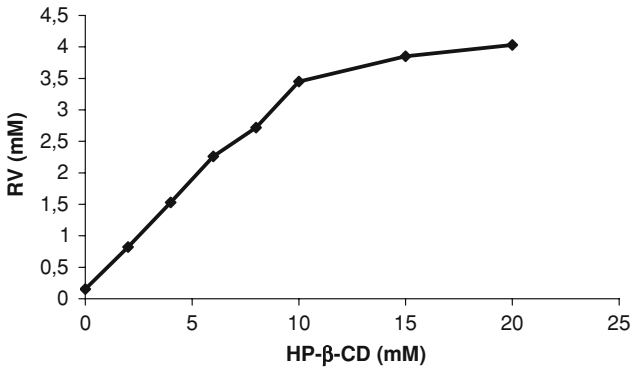

Fig. 2 Solubility curve of *trans*-resveratrol in buffered solution at pH 6.0 as a function of increasing concentrations of HP-β-CD

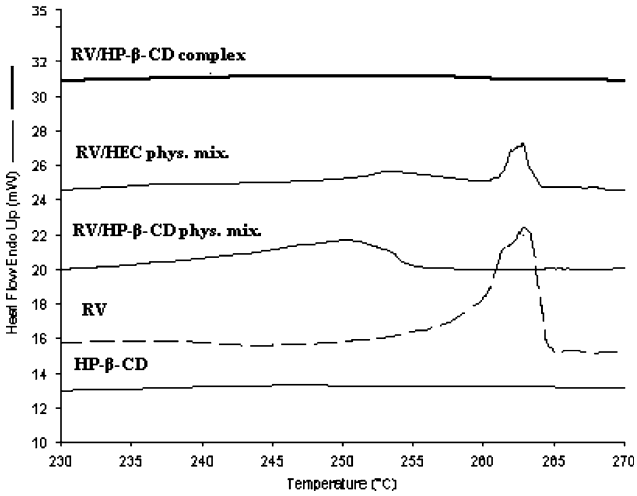

Fig. 3 Thermograms of HP-β-CD, RV, RV/HP-β-CD physical mixture, RV/HEC physical mixture and RV/HP-β-CD complex

complexes are preferred whereas other authors characterized RV/CD complexes by lipoxygenase inhibition [24]. In both cases the apparent formation constants were of the same order of magnitude as that described in this work.

DSC studies

The DSC thermograms gave further information about the interaction between RV and HP-β-CD. Firstly, no signal was observed below 200 °C (data not shown); DSC curve of pure RV displayed an endothermic peak at 263.53 °C, while the thermograms of HP-β-CD did not show any peak (Fig. 3). The disappearance of melting peak in the thermogram of the complex indicated the presence of an interaction between the two species. In fact the peak of RV was not detected since the crystalline active molecule was inside the cavity of the cyclodextrin. In the thermogram of the RV/HP-β-CD physical mixture the shape and the shift of the peak indicated the existence of a new solid phase in which the RV was dispersed with a lower crystallinity than pure RV. The thermogram of the physical mixture with a non-complexing matrix (HEC), showed RV melting peak around 260 °C which was not present in case of RV/HP-β-CD physical mixture thus further confirming the complex formation with the cyclodextrin.

Photodegradation studies

RV absorbs in the UV region, with a peak at 306 nm, as above reported and upon UVA irradiation the peak

decreases as a *cis-trans* isomerisation occurs, indicating a photolysis process of the molecule. In this study the irradiation runs were carried out separately on pure RV and on RV/HP-β-CD complex, in order to investigate the protective effect of the cyclodextrin on the photodegradation of the active molecule. The ability of cyclodextrins to protect some active substances against photodecomposition of some photosensitive compounds have been recently reported [17, 25].

In the present work the influence of the medium (ethanol, gel, gel emulsion, O/W emulsion or liposomes) was also examined and it has been observed that in all of them RV degraded under UVA light following a first order kinetic, as confirmed by the curves obtained reporting the concentration in function of irradiation time.

The first medium considered in the present paper was a 2% HEC gel (Fig. 4).

The curves of photolysis were linearized ($R^2 > 0.991$) in a logarithmic form to obtain the following general equation:

$$\ln\left[\left(C_t - C_{inf}\right)/\left(C_0 - C_{inf}\right)\right] = kt$$

where C_0 is the initial RV concentration, C_t the concentration of RV at time t, C_{inf} is the concentration of RV at infinite time, k is the degradation rate constant and t is the irradiation time, in minutes. In a second series of experiments increasing amounts (10, 30 and 50% w/w) of absolute ethanol were added to HEC gel. Also in these cases the degradation of the molecule followed a first order kinetic. In Table 2 the kinetic constants of photolysis rate are listed.

Comparisons between the kinetic constant values of Table 1 underlined that by increasing the percentage of ethanol in the gel the photodegradation rate of RV decreased. This finding suggests a certain protective effect of ethanol against UV photodegradation of the active substance. In our previous paper [26] it has been reported that environments with low polarity (hydroalcoholic mixtures) are able to inhibit the charge transfer reactions that cause substrate photooxidation. So also in this case we can

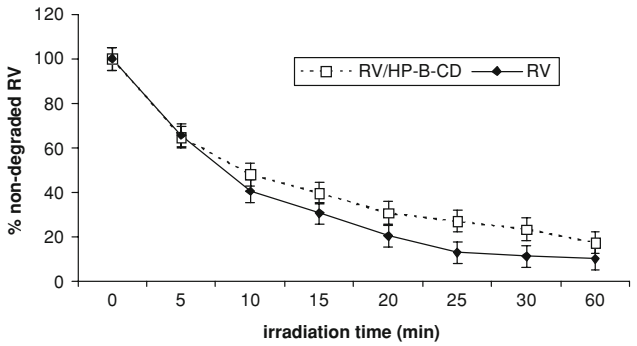

Fig. 4 Curves of photodegradation of RV (0.2 mM), free and complexed with HP-β-CD, in HEC gel

Table 2 Constants of photodegradation rate of RV (0.2 mM) in HEC gel containing different amounts of ethanol

HEC gel/ethanol	k_c (s^{-1})
50/50	$1.32\ (\pm 0.08) \cdot 10^{-3}$
70/30	$1.56\ (\pm 0.09) \cdot 10^{-3}$
90/10	$1.65\ (\pm 0.07) \cdot 10^{-3}$
100/0	$1.66\ (\pm 0.09) \cdot 10^{-3}$

suppose that RV photolysis process involves charge transfer reactions, such as photoionization or heterolytic bond cleavage which are usually favored in more polar media.

To extent the study, the photodegradation of RV was performed in all the media by comparing the time evolution of free RV with that of complexed RV at the same concentration.

A comparison of the obtained kinetic constants (see Table 3) indicates that in all the considered media the inclusion in HP-β-CD slightly decreased the photodegradation rate of the guest molecule. Our hypothesis is that, when RV was enclosed in the hydrophobic cavity it experienced an apolar environment that should reduce the photolytic reaction. On the other hand the small protective effect could be due to some screening effect of CD particles for the passage of light to reach the guest molecule [25].

Interestingly, the addition of BHT to the emulsion slightly decreased RV photodegradation rate but this protective effect was lower than that offered by the complexation phenomenon.

Finally, the photodegradation study of RV has also been performed in liposomes to evaluate if these vesicles can give a certain protection to the active. The amount of RV incorporated into liposomes was always in the 95–98% range of that initially dissolved in the organic phase suggesting a complete location in the lamellar structure of the vesicles. The first system under study was the aqueous dispersions of liposomes in which the active was entrapped at 2.0 mM concentration. Next, the experiment was carried out on liposomes dispersed (1:10 ratio) in gel, gel emulsion

Table 3 Constants of photodegradation rate of RV (0.2 mM) in different media

Medium	k_c (s^{-1})	
	Free RV	RV/HP-β-CD
HEC gel	$1.66\ (\pm 0.08) \cdot 10^{-3}$	$1.36\ (\pm 0.09) \cdot 10^{-3}$
Gel emulsion	$0.71\ (\pm 0.07) \cdot 10^{-3}$	$0.65\ (\pm 0.10) \cdot 10^{-3}$
O/W emulsion	$1.16\ (\pm 0.10) \cdot 10^{-3}$	$0.80\ (\pm 0.07) \cdot 10^{-3}$
O/W emulsion + BHT (0.005% w/w)	$1.04\ (\pm 0.05) \cdot 10^{-3}$	–

or O/W emulsion. In Table 4 the photodegradation rate constants of RV entrapped in liposomes are reported.

The first observation is that when RV was included in liposomes degraded with a rate that increases following this sequence: aqueous dispersion <dispersion in gel emulsion <dispersion in HEC gel <dispersion in O/W emulsion. The kinetic constant values of Table 4 show that the lowest rate of degradation was noted in those liposomes that were dispersed in gel emulsion. Furthermore, comparisons between Tables 3 and 4 indicated that in all the considered media RV degraded slightly faster when it is pure than when it is entrapped in liposomes. On the other hand it is well known that phospholipids play an important role in the protection of some oxidable substrates against autooxidation [27].

Antiradical activity of RV

DPPH$^{\cdot}$ is widely used for assessing the ability of polyphenols to transfer labile H atoms to radicals, a common mechanism of antioxidant protection [19, 28]. Our data (Fig. 5) suggest that DPPH$^{\cdot}$ absorbance decreased by increasing RV concentration indicating a direct interaction between the two compounds and resulting in a significant antiradical activity of RV, comparable to that observed with Trolox, a well-known antiradical compound [26].

Furthermore, comparisons between complexed RV and free RV evidenced that the inclusion phenomenon did not significatively limit the antiradical activity of this compound.

Table 4 Constants of photodegradation rate of RV entrapped in liposomes dispersed in different vehicles

RV-loaded liposomes	k_c (s^{-1})
In water	$0.56\ (\pm 0.04) \cdot 10^{-3}$
In HEC gel	$1.06\ (\pm 0.08) \cdot 10^{-3}$
In gel emulsion	$0.67\ (\pm 0.04) \cdot 10^{-3}$
In O/W emulsion	$1.10\ (\pm 0.10) \cdot 10^{-3}$

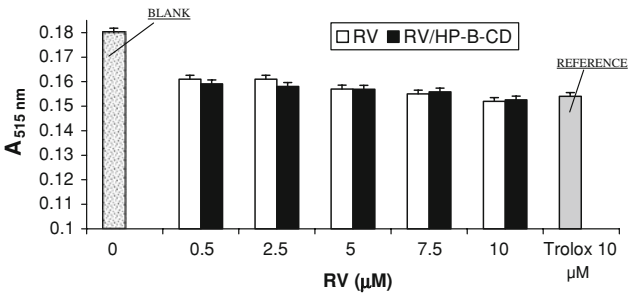

Fig. 5 Antiradical activity of RV and of RV/HP-β-CD represented by the decrease of DPPH$^{\cdot}$ absorbance as a function of increasing antioxidant concentration

Metal-chelating properties of RV

The ability of RV to form complexes with metal ions has been previously discussed in the literature [29]. In this study the chelating properties of RV, free and complexed with HP-β-CD, were compared to verify if the inclusion in the cavity of the cyclodextrin could interfere with this activity. The analysis was carried out using the method of competitive replacement. The addition of $CuCl_2$ to RV solution resulted in the decrease of the peak absorbance at 306 nm (Fig. 6).

In the case of RV/HP-β-CD complex the absorbance alterations followed a trend similar to that observed with free RV suggesting that the active molecule exercised its chelating activity also when it is included in the cavity of the host molecule.

In vitro skin accumulation studies

Since RV is frequently employed topically as antioxidant and also for skin cancer prevention [8, 9] it can be interesting to study in vitro its skin absorption. This experiment allowed to determine the amount of RV, pure or complexed, which accumulates in the porcine skin. The method used was in line with the guidelines published by Diembeck et al. [30]. A negligible percutaneous permeation within 24 h was observed in all the receiving phases while the accumulation data in porcine ear skin 24 h following application are presented in Table 5. By comparing the two

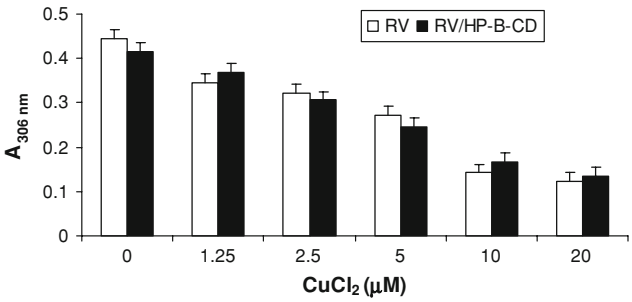

Fig. 6 Absorbance of free and complexed RV (306 nm) as a function of increasing concentrations of $CuCl_2$

Table 5 RV accumulation in porcine skin 24 h following application

Sample	Accumulation ($\mu g/cm^2$)
RV in ethanol	1.98 ± 1.5
RV/HP-β-CD in gel emulsion	4.05 ± 1.8
RV/HP-β-CD in HEC gel/Oramix CG110 mixture	4.78 ± 1.7

formulations containing RV/HP-β-CD complex with the reference ethanol solution of free RV it can be observed that HP-β-CD increased up to two folds the skin accumulation. On the other hand cyclodextrins are known to enhance drug permeation by increasing its solubility and acting as a carrier of drug from the formulation towards the lipophilic surface of the skin, where the drug partitions from the complex into the skin [31].

Assay of peroxidation in porcine skin

UV exposure of the skin has been shown to produce excessive generation of reactive oxygen species (ROS) which if remain uncounteracted by the antioxidant defense ability of the living system, creates a situation of oxidative stress in the skin. This stress is shown to be responsible for a variety of pathological conditions including inflammation, aging, immunosuppression and skin cancer [8]. Therefore, prevention with antioxidant should offer a strategy for the management of these oxidative stress conditions. In the recent years, the naturally occurring agents present in the common diet consumed by human population, have gained considerable attention.

This experiment was designed to assess the preventive effects of RV against the damages of UVA radiations to the porcine skin. Because lipoperoxidation is regarded as a marker of oxidative stress, we evaluated the protective effect of RV on UVA-induced skin lipoperoxidation by monitoring the formation of MDA from the skin substrate. Figure 7 shows that UVA exposure of the skin resulted in a significant increase in MDA levels indicating an increase in lipoperoxidation phenomenon. The presence of RV was found to inhibit the lipoperoxidation activity both in the dark and under UVA irradiation. Furthermore, this inhibiting effect was observed also for RV/HP-β-CD complex. In brief this finding demonstrated that the complexation of RV with HP-β-CD did not significantly limit its antioxidant activity.

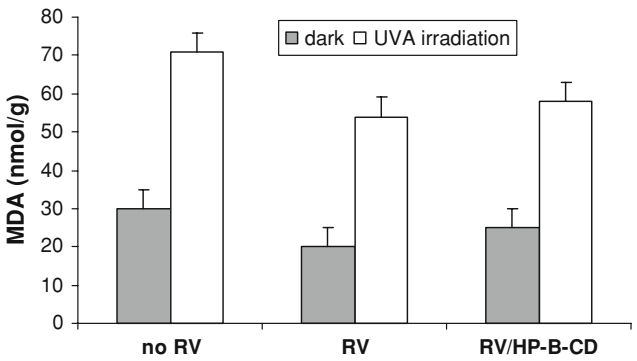

Fig. 7 MDA levels derived from 1 gram of porcine skin in the absence and in the presence of RV in dark conditions and under UVA irradiation

Conclusions

In this paper an inclusion complex between *trans*-resveratrol and HP-β-CD was investigated. Computational studies, phase diagrams and DSC thermograms suggested the formation of the inclusion complex. The present investigation underlines the possibility of increasing the photostability of *trans*-resveratrol by including it in HP-β-CD. The photoprotection was more evident in gel emulsion than in hydrophilic media where photolysis reactions are favored. Nevertheless it was found that the location of resveratrol inside the cavity of the host molecule did not significatively limit its antioxidant and metal-chelating properties. Furthermore in vitro studies demonstrated that the complexation phenomenon enhanced the accumulation of resveratrol in porcine skin. Taken together, these data suggest that the complex resveratrol/HP-β-CD could play a primary role in protection against oxidative stress of skin and thus might have application potential.

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