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A characterization study of resveratrol/sulfobutyl ether-β-cyclodextrin inclusion complex and *in vitro* anticancer activity

Valentina Venuti[a], Carmela Cannavà[b], Maria Chiara Cristiano[c], Massimo Fresta[c], Domenico Majolino[a], Donatella Paolino[c], Rosanna Stancanelli[b], Silvana Tommasini[b], Cinzia Anna Ventura[b,*]

[a] Dipartimento di Fisica e di Scienze della Terra, Università degli Studi di Messina, Viale Ferdinando Stagno D'Alcontres 31, I-98166 Messina, Italy
[b] Dipartimento di Scienze del Farmaco e Prodotti per la Salute, Università degli Studi di Messina, V.le Annunziata, I-98168 Messina, Italy
[c] Dipartimento di Scienze della Salute, Università Magna Graecia di Catanzaro, Viale Europa, I-88100 Catanzaro, Italy

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ABSTRACT

A resveratrol/sulfobutylether-β-cyclodextrin inclusion complex was prepared using the freeze-drying method and characterized in solution through UV–vis spectroscopy, solubility phase studies and Job's plot methods. At the solid state it was characterized using the FTIR-ATR technique. Sulfobutylether-β-cyclodextrin has a high affinity for the drug, and forms an inclusion complex with a 1:1 molar ratio both in solution and as a solid sample. It also has a high stability constant (K_c, 10,114 M^{-1}). Complexation strongly increases the water solubility of resveratrol (from 0.03 mg/ml to 1.1 mg/ml, at 25 °C) and positively influences its *in vitro* anticancer activity which was observed on a human breast cancer cell line (MCF-7). In solid phase, FTIR-ATR revealed itself as being a useful technique in elucidating the complexation mechanism, which it did by emphasizing the functional groups involved in the activation of non-covalent "host–guest" interactions.

1. Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene – RSV) is a triphenolicphytoalexin found in a variety of plant species [1]. The phenolic nature of RSV explains its antioxidant activity. It has been shown to provide health-promoting benefits, such as lowering the incidence of coronary heart disease, and it possesses qualities that prevent cancer. It also manifests estrogenic activity with varying degrees of estrogen receptor agonist due to its structure which is similar to the synthetic estrogen diethylstilbestrol [2]. In the area of chemoprevention, a great number of studies have been performed, demonstrating that RSV acts through numerous mechanisms, including the regulation of cell cycle progression [3] and apoptosis [4], the inhibition of tumor invasion and angiogenesis [5], the prevention of inflammation [6], the activation of adenosine monophosphate-activated protein kinase (AMPK) [7], the scavenging of reactive oxygen species [8] and the modulation of NFkB [9]. This activation of multiple anti-cancer pathways (pleiotropism) is an attractive feature of RSV, since it may help to overcome drug resistance [10]. It has been suggested that RSV is capable of mediating a great number of other biological responses relevant to human health such as protection against viral infections [11] and caloric restriction mimicry [12], as well as having beneficial effects on longevity [13].

RSV is a solid off-white powder soluble in ethanol and in dimethyl sulfoxide but it is practically insoluble in water (~0.03 mg/ml at 25 °C) according to the European Pharmacopeia definition, and its log *P* is 3.1 [14]. The drug exists as two structural isomers: cis-(Z) and trans-(E) (Fig. 1). The trans-isomer is biologically more active than the cis-isomer [15], probably due to its non-planar conformation. When protected from light, trans-RSV is stable for at least 42 h and for at least 28 days in pH 1 and 7 buffers, respectively; whereas the *cis* form is only stable at neutral pH when completely shielded from light [16].

The great hydrophobicity of RSV constitutes a serious problem for its oral bioavailability and for the realization of liquid formulations, it requires the use of solvents that might not be suitable for parenteral administration. So despite its promising beneficial effects, the clinical use of RSV is very limited. To date, it is present on the market only as a nutraceutical product. The realization of fast-dissolving-solid formulations or free solvent–liquid formulations of RSV could open new perspectives for

* Corresponding author. Tel.: +39 090 6766508; fax: +39 090 6766402.
E-mail address: caventura@unime.it (C.A. Ventura).

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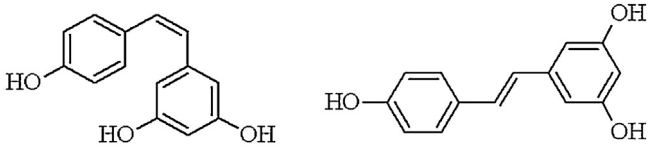

cis-resveratrol *trans*-resveratrol

Fig. 1. Chemical structures of cis- and trans-resveratrol.

the pharmaceutical use of this active compound, that could show *in vivo* anticancer activity comparable to other natural products. To this end, cyclodextrins (Cyds) can be used. Compounds that are poorly soluble in water and hydrophobic moieties of amphiphilic molecules can interact non-covalently with Cyd cavities to form the so-called inclusion complexes, which are generally highly water-soluble [17–21]. The solubility of these complexes depends principally on the type of Cyd used. Hydroxypropyl-β-cyclodextrin (HP-β-Cyd) and sulfobutylether-β-cyclodextrin (SBE-β-Cyd) are non-toxic and biocompatible Cyd derivatives which exhibit solubility and complexing abilities greater than those of the parent Cyd [22]. In particular, SBE-β-Cyd interacts very well with neutral drugs to facilitate solubility and chemical stability, and because of its polyanionic nature, it interacts particularly well with cationic drugs. Moreover, its four-carbon butyl chain coupled with the repulsion of the end group's negative charge allows for an extension of the SBE-β-Cyd cavity, thus binding a drug more strongly, as compared to other modified Cyds [22].

Researchers have recently been studying the inclusion complex of RSV with natural or modified Cyds [23], and they report an increase of water solubility and of the antioxidant activity of the drug, particularly when it is complexed with HP-β-Cyd [24,25]. Complexation of RSV with SBE-β-Cyd has been less studied and to our knowledge no detailed characterization studies of this complex are present in literature.

In this work we prepared the inclusion complex of RSV with SBE-β-Cyd and the influence of this on the water solubility of the drug was evaluated. The complex was prepared by the freeze-drying method and characterized in the solid state by Fourier transform infrared spectroscopy in attenuated total reflectance (FTIR-ATR) geometry. In solution, solubility phase studies and Job's plot were performed in order to investigate the stoichiometry of the inclusion complex. *In vitro* biological assays on a human breast cancer cell line (MCF-7) were performed to evaluate the influence of SBE-β-Cyd on the anticancer activity of RSV.

2. Materials and methods

2.1. Materials

Resveratrol (3,5,4′-trihydroxystilbene, $C_{14}H_{12}O_3$, MW 228.24) (RSV) is a product of Sigma–Aldrich Chemie® (Italy); sulfobutylether-β-cyclodextrin (CAPTISOL®, average degree of sulfobutyl substitution: seven; average MW 2162) (SBE-β-Cyd) was kindly supplied by CyDex Pharmaceutical (Lenexa, Kansas City, USA). They were all employed without any further purification. The water used throughout the study was double-distilled and de-ionised, then filtered through 0.22 μm Millipore® GSWP filters (Bedford, USA). Dulbecco's modified eagle's medium (DMEM) is a Life Technologies product (Milan, Italy). Fetal bovine serum (FBS), glutamine, penicillin, streptomycin and trypsin/EDTA (1×) solution were purchased from Invitrogen Corporation (Giuliano Milanese, Milan, Italy). 3-[4,5-Dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide (MTT) dye test (TLC purity 97.5%), phosphate buffer (PBS) solution and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (Milan, Italy). MCF-7 cells were provided by IZS of Modena and Reggio-Emilia.

2.2. Preparation of the inclusion complex

SBE-β-Cyd (200 mg) was solubilized in 10 ml of water at room temperature and added to an amount of RSV exceeding its intrinsic solubility. The flask was repaired from the light in order to prevent the conversion of the RSV from the trans- to the cis-position, sonicated in a Bandelin RK 514 water bath (Berlin, Germany) for 30 min, then stirred for 3 days. Then the suspension was filtered through Sartorius Minisart®-SRP 15 PTFE 0.22 μm filters (Germany) and freeze-dried (VirTis Gardiner, USA BenchTop K Series Freeze Dryers).

2.3. Phase-solubility analysis

Phase-solubility studies were performed using the method described by Higuchi and Connors [26]. An amount of RSV exceeding its solubility was added to unbuffered aqueous solutions of SBE-β-Cyd ($0.0–8.0 \times 10^{-3}$M) in 5 ml capped tubes, then sonicated in a Bandelin RK 514 water bath (Berlin, Germany) for 15 min. The flasks were sealed to avoid changes due to evaporation and magnetically stirred for 3 days in a Telesystem stirring bath thermostat 15.40 with a Telemodul 40 C control unit at 25 ± 0.1 °C. After equilibrium was reached, the suspensions were filtered through Sartorius Minisart®-SRP 15 PTFE 0.22 μm filters (Germany) and analyzed through UV–vis spectroscopy, in the 200–400 nm spectral range (FullTech Instruments double beam spectrophotometer, mod. PG T80, Italy). This was done to evaluate the amount of RSV dissolved. All measurements were repeated at least three times. The data obtained were used to determine the binding constant of the RSV–SBE-β-Cyd inclusion complex, according to Higuchi and Connors equations [26]. No degradation of RSV was observed under experimental conditions.

2.4. Job's plot method

Equimolar (1×10^{-5}M) methanol/water solutions (55/45, v/v) of RSV and SBE-β-Cyd were mixed to a fixed volume by varying the molar ratio from 0.1 to 0.9, keeping the molar concentration of the species constant. After stirring for 1 h, the absorbance of each solution was measured by UV–vis spectroscopy at 305 nm and Δabs was determined as the difference between abs without and with Cyd. Then, Δabs × [RSV] was plotted *vs.* R (R = [RSV]/[RSV] + [SBE-β-Cyd]) [27].

2.5. Water solubility and dissolution rate determination

The degree of water solubility of the free RSV and the RSV/SBE-β-Cyd inclusion complex was determined by suspending excess amounts of each sample in 3 ml of water and stirring at 25 ± 0.1 °C for 2 days. The suspensions were then filtered through Sartorius Minisart®-SRP 15 PTFE 0.22 μm filters (Germany) and analyzed by UV–vis spectroscopy at 305 nm.

The determination of the dissolution rates of the same samples was carried out according to the USP 32nd paddle method. Three hundred and thirty mg of free RSV or a corresponding amount in complex were suspended in 900 ml of water and stirred at 100 rpm at 37 ± 0.5 °C. At fixed intervals the concentration of RSV in solution was assayed by UV–vis spectroscopy at 305 nm. The medium was reconstituted with fresh water and the data were corrected for the operated dilution. The experiments were carried out in triplicate and data were presented as mean ± standard deviation.