THEMATIC RESEARCH

SULFATION PATHWAYS

Potential benefits of a sulfated resveratrol derivative for topical application

Marta Correia-da-Silva1,2,*, Verónica Rocha3,4,*, Cláudia Marques3, Cláudia M Deus4,2, Adriana Marques-Carvalho4, Paulo J Oliveira4,5, Andreia Palmeira1,2, Madalena Pinto1,2, Emília Sousa1,2, José Manuel Sousa Lobo3 and Isabel Filipa Almeida3

1Laboratory of Organic and Pharmaceutical Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal
2CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, Matosinhos, Portugal
3UCIBIO, REQUIMTE, Laboratory of Pharmaceutical Technology, Department of Drug Sciences, Faculty of Pharmacy University of Porto, Porto, Portugal
4CNC – Center for Neuroscience and Cell Biology, University of Coimbra, UC Biotech Building, Biocant Park, Cantanhede, Portugal
5IIIUC – Institute for Interdisciplinary Research, University of Coimbra, Coimbra, Portugal

Correspondence should be addressed to E Sousa: esousa@ff.up.pt

*(M Correia-da-Silva and V Rocha contributed equally to this work)

This paper is part of a thematic section on Sulfation Pathways. The guest editors for this section were Jonathan Wolf Mueller and Paul Foster.

Abstract

Resveratrol (RSV) is a polyphenolic compound with antioxidant, anti-inflammatory and anti-aging properties partly associated with sirtuin 1 (SIRT1)-activation in the skin. However, poor water solubility may limit RSV efficacy. This work aimed to clarify the interest of a new synthetic water-soluble RSV derivative (resveratrol glucoside sulfate, RSV-GS) for topical application. Resveratrol glucoside sulfate was synthesized using microwave-assisted sulfation. Cytotoxicity assays were performed with the keratinocyte HaCaT cell line, using MTT reduction, neutral red uptake, Alamar Blue/resazurin reduction, trypan blue exclusion and measurement of ATP concentration. Western blotting was used to evaluate SIRT1 protein content. Regarding SIRT1 binding, an in silico docking study was performed, using AutoDock Vina. Our results showed that the synthetic derivative RSV-GS was 1000 times more soluble in water than RSV and its non-sulfated glucoside. No relevant decrease in HaCaT cell viability was observed for concentrations up to 5 mM for RSV-GS, and up to 500 μM for resveratrol glucoside, while a significant decrease in HaCaT viability occurred from 100 μM for RSV. RSV-GS and RSV showed a similar behavior regarding protective effect against oxidative stress-induced cytotoxicity. SIRT1 protein content increased after treatment with 500 μM of RSV-GS and 100 μM of RSV. Moreover, in silico studies predicted that RSV-GS binds more stably to SIRT1 with a lower binding free energy than RSV. Although these results support the possible use of RSV-GS in topical formulations, in vivo safety and efficacy studies are needed before considering the use of RSV-GS in commercial products.

Introduction

Resveratrol (RSV), 3,5,4′-trihydroxystilbene, is a polyphenolic compound found in the seeds and skin of grapes, red wine, peanuts and berries (Kasiotis et al. 2013).
is a phytoalexin (Amri et al. 2012, Tang et al. 2014). The most abundant form of RSV in nature is resveratrol 3-O-β-D-glucoside (RSV-G) (Regev-Shoshani et al. 2003), also called piceid or polydatin.

Several studies demonstrated that RSV has interesting properties regarding skin care (Baxter 2008, Ndiaye et al. 2011, Park et al. 2014). The biological effects of RSV are supported by mechanistic data that indicates the involvement of increased expression and activation of the protein deacetylase sirtuin 1 (SIRT1) (Baur & Sinclair 2006, Price et al. 2012). Sirtuin 1 is expressed in skin keratinocytes (Blander et al. 2009). Resveratrol, acting as a SIRT1 activator, protects the skin against oxidative stress events and consequently, prevents skin cell damage (Cao et al. 2009).

Resveratrol occurs in cis-(Z) and trans-(E) isomeric forms in nature. trans-resveratrol is the most common and biologically active isomeric form as well as the most photo- and thermal-stable isomer (Baxter 2008, Carlotti et al. 2012). Fast trans-cis isomerization (Detoni et al. 2012, Sanna et al. 2012) and poor water solubility (Lepak et al. 2015) limits RSV efficacy and constrains the design of topical formulations. Besides classical formulation development (Amri et al. 2012, Summerlin et al. 2015), chemical conjugations, such as sulfation (Hoshino et al. 2010), glycosylation (Regev-Shoshani et al. 2003), oligomerization (Matsuura et al. 2015) and other derivations (Regev-Shoshani et al. 2003, Medina et al. 2010, Mattarei et al. 2013) are being employed to overcome these limitations.

Synthetic RSV derivatives with improved water solubility may be obtained with similar or better activities toward SIRT1 activation. In this work, the validation of a synthetic water-soluble RSV derivative (resveratrol glucoside sulfate, RSV-GS) for skin care was evaluated and compared with the parent compounds RSV and RSV-G, by studying the cytotoxic and protective effects (lysosomal integrity, cell membrane damage and metabolic activity) and the alterations of SIRT1 protein content using a human keratinocyte cell line and performing in silico affinity studies for SIRT1.

**Materials and methods**

**Reagents**

Dulbecco’s Modified Eagles’s Medium (DMEM) with 25 mM D-glucose and 1 mM pyruvate, inactivated fetal bovine serum (FBS), penicillin-streptomycin solution, Dulbecco’s phosphate buffered saline (DPBS) without calcium chloride and magnesium chloride, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution were supplied by Gibco by Life Technologies. Dimethyl sulfoxide (DMSO), 0.4% trypan blue solution, neutral red (NR) solution and resazurin sodium salt were purchased from Sigma-Aldrich. AlamarBlue was provided by Thermo Scientific. 3-[4,5-Dimethyl-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was acquired from Life Technologies. Sirtuin-1 and actin antibodies were obtained from Abcam and Millipore, respectively. In all experiments, purified water was used and obtained using a Direct-Q Water Purification System (Merck Millipore) with a reverse osmosis process. trans-Resveratrol was purchased from Fragon (São Paulo, Brazil), trans-resveratrol 3-β-D-glucopyranoside (572691) and triethylamine sulfur trioxide adduct ($ 5139) were purchased from Sigma-Aldrich. UV/Vis spectra were traced with a synergy HT microplate reader from BioTek with GEN5 software. Melting point was obtained in a Köfler microscope and was not corrected. Infrared (IR) spectra were recorded on a Nicolet iS10 from Thermo Scientific with Smart OMNI-Transmission accessory (Software 188 OMNIC 8.3) in KBr. Nuclear magnetic resonance (NMR) spectra were taken in DMSO-d$_6$ at room temperature, on Bruker AMC AVANCE 300 instrument (Bruker Biosciences Corporation, Billerica, MA, USA) instrument. High-resolution mass spectrometry (HRMS) results were obtained in CACTI services, Vigo, Spain.

**Synthesis of resveratrol glucoside sulfate (RSV-GS)**

A mixture of trans-resveratrol 3-β-D-glucopyranoside (0.5 g, 1.28 mmol) and triethylamine sulfur trioxide adduct (4 equiv/OH) in dimethylacetamide (10 mL) was stirred and heated for 30 min at 100°C under MW irradiation (200W). After cooling, the mixture was poured into acetone (200 mL) under basic conditions (triethylamine) and left at 4°C for 24 h. The formed crude oil was washed with ether, dissolved in a minimum volume of water and applied on a DOWEX cation exchange resin (Na$^+$ form). After evaporation until dryness, a light brown solid was obtained (85% yield). NMR and HRMS were according to previously reported data (Correia-da-Silva et al. 2011).

**Water solubility determination**

The solubility was tested at the maximum solubility class of the European Pharmacopeia (very soluble, >1000 mg/mL) by dissolving a given amount in ultra-pure water (UPW) (RSV and RSV-G: 2 mg in 200 mL; RSV-GS: 50 mg in...
50µL) and stirring at 25°C for 1 h. After visual inspection, RSV-GS was the only soluble compound. Suspensions of RSV and RSV-G were filtered through a 0.22µm membrane filter with a syringe. After filtration, 200µL of each solution was transferred to a microplate well and a 1:10 dilution was made. The UV/Vis Spectra were traced at 310 and 340 nm for RSV and RSV-G, respectively, and the concentration was determined according to the standard calibration curve obtained for each compound. Three standard calibration curves were obtained with 4 different concentrations for each compound (RSV: 0.010, 0.005, 0.0010 and 0.0005 mg/mL; RSV-G: 0.050, 0.025, 0.005 and 0.0025 mg/mL). The solubility assay was repeated in three independent days.

**Cell culture conditions**

The immortalized human keratinocyte cell line (HaCaT) was obtained from Cell Lines Service (CLS, Eppelheim, Germany) (Boukamp et al. 1988). The method of authentication involved characterization of DNA Profile (STR). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in Dulbecco’s modified Eagle’s medium (D31966) supplemented with 10% FBS, 2 mM glutamine and 1% penicillin/streptomycin (growth medium). HaCaT cell line was bought from the vendor at passage number 31 and experiments were performed after that passage.

**Cytotoxicity testing**

Cytotoxicity was evaluated using different assays, namely MTT reduction, NR uptake, Alamar Blue reduction and trypan blue exclusion. HaCaT cells were incubated with different concentrations of each compound (RSV and RSV-G: 25, 50, 100, 350, and 500µM; RSV-GS: 100, 350, 500, 1000 and 5000µM). Cytotoxicity assessment of RSV in this cell line was already published by our group (Rocha et al. 2017), but the results were reproduced here for comparison purposes. For the trypan blue assay, the concentration range used was slightly different and is described below. DMSO (10%) was used as positive control and cells treated with the solvent as negative control. Cell viability was calculated with regard to negative control, which was set to 100% viability. IC₅₀ was calculated by linear regression. All absorbance readings were performed on a multi-mode microplate reader (Synergy HT BioTek Instruments). A minimum of three independent experiments were performed for each assay.

**MTT reduction assay**

HaCaT cells (1 × 10⁴ cells/well, 96-well microplates) were incubated with different concentrations of RSV, RSV-G or RSV-GS. After 24-h incubation, cells were washed with DPBS and 0.5 mg/mL MTT solution (serum free DMEM) was added. After 2-h incubation, DMSO was added to dissolve the formazan crystals, and absorbance was measured at 570 nm.

**Neutral red uptake assay**

HaCaT cells (2 × 10⁴ cells/well, 96-well microplates) were incubated with different concentrations of RSV, RSV-G or RSV-GS. After 24-h incubation, NR solution (DMEM) was added (50µg/mL) to cells previously washed with DPBS. Cells were washed with DPBS, after 3 h of incubation, and a solution of 50% ethanol/1% acetic acid was added to extract the NR dye captured within the cells. The plates were placed for 10 min in a microplate shaker, at room temperature and protected from light. Absorbance was measured at 540 nm.

**Trypan blue exclusion assay**

HaCaT cells (1 × 10⁵ cells/well, 12-well microplates) were incubated with different concentrations of RSV, RSV-G or RSV-GS (RSV and RSV-G: 25, 200 and 500µM; RSV-GS: 100, 500 and 5000µM). After 24 h of incubation, cells were trypsinized and counted using trypan blue vital dye (1:1).

**Measurement of cell protection against oxidative stress**

Protective effects of RSV and derivatives against H₂O₂- and menadione-induced cytotoxicity were evaluated using resazurin assay and ATP levels determination. HaCaT cells were seeded as described in each protocol described below and incubated with different concentrations of each compound (RSV and RSV-G: 25, 50, 100, 350 and 500µM; RSV-GS: 100, 350, 500, 1000 and 5000µM) during 24 h. Vehicle controls received an equivalent amount of DMSO only, which never exceeded 0.1% v/v. Afterward, 9 mM H₂O₂ or 25 µM menadione were added during 3 h. A minimum of four independent experiments were performed for each assay.

**Sulforhodamine B (SRB) assay**

The SRB assay was used to detect the loss of cell mass resulting from H₂O₂- or menadione-induced toxicity. Cells were seeded at a concentration of 20,000 cells/cm²
in 96-well plates, with a final volume of 100 µL per well. Cells were treated with 7.8, 15.6, 31.2, 62.5, 125 and 250 µM menadione and 1.9, 3.9, 7.8, 15.6 and 31.2 mM H₂O₂ during 3 h. At specific time points, the incubation medium was removed, and cells were fixed in 1% acetic acid in ice-cold methanol for at least 1 day. Cells were then incubated with 0.05% (w/v) SRB reagent dissolved in 1% acetic acid for 1 h at 37°C. Unbound dye was removed with 1% acetic acid. Dye bound to cell proteins was extracted with 10 mM Tris-base solution, pH 10. After SRB labeling, absorbance was measured in a BioTek Cytation 3 spectrophotometer at 510 nm and the amount of dye released is proportional to the number of cell mass in each well (Vichai & Kirtikara 2006).

Alamar Blue/resazurin assay
HaCaT cells (2 × 10⁴ cells/well, 96-well microplates) were incubated with different concentrations of RSV, RSV-G or RSV-GS. After 24-h incubation, 10% AlamarBlue was added to each well and incubated for 4 h. Finally, the absorbance at 570 and 600 nm was determined.

The resazurin assay was used to measure the cellular viability based on metabolic activity of living cells through the fluorescence intensity. Cells were seeded at a concentration of 20,000 cells/cm² in 96-well plates, with a final volume of 100 µL per well. Resazurin stock solution (1 mg/mL) was prepared in PBS 1× and stored at −20°C. Cells were treated as described in protective testing section. At specific time points, cell media from each well was removed carefully and cells were washed with PBS 1×. After removing the PBS 1×, 100 µL of resazurin solution (1:100 dilution in growth medium from a stock solution) was added to each well and incubated for 4 h at 37°C with a 5% CO₂ atmosphere. Resorufin fluorescence was measured in a BioTek Cytation 3 spectrophotometer using excitation wavelength of 540 nm and emission of 590 nm (Silva et al. 2016). The spectral characteristics of DMEM and resazurin and menadione (25 µM) were determined in 96-well plate by BioTek Cytation 3 spectrophotometer using wavelength between 350 and 700 nm (visible spectrum).

ATP levels determination
Intracellular ATP levels were measured by using CellTiter-Glo Luminescent Cell Viability Assay (Promega) following manufacturer’s instructions. Cells were seeded at a concentration of 20,000 cells/cm² in white opaque-bottom, 96-well plates, with a final volume of 100 µL per well. Cells were treated as described in protective testing section. At specific time points, 50 µL of CellTiter-Glo Reagent (CellTiter-Glo Buffer + CellTiter-Glo Substrate) was added to the cells. Contents were mixed for 2 min on an orbital shaker to induce cell lysis and, after 10 min of incubation at 22°C, the luminescence signal was monitored in a BioTek Cytation 3 spectrophotometer (BioTek Instruments). ATP standard curve was also generated following manufacturer’s instructions. The luminescence signal obtained was proportional to the amount of ATP present in solution.

Protein semi-quantification by western blotting
HaCaT cells were treated with two concentrations of each compound (RSV and RSV-G: 25 and 100 µM; RSV-GS: 100 and 500 µM) during 24 h. Vehicle controls only received an equivalent amount of DMSO, which never exceeded 0.1% v/v. To obtain total cellular extracts, all cells were harvested by trypsinization and washed with PBS 1×. In order to collect the cells, one centrifugation step was performed for 5 min at 1000 g (4°C). The cellular pellet was resuspended in cell lysis buffer 1× (Bio-Rad, 9803) supplemented with 100 µM phenylmethylsulfonyl fluoride (PMSF). Protein content was determined by the Bradford method using bovine serum albumin as a standard (Bradford 1976). An equivalent amount of protein (20 µg) for each sample was separated by electrophoresis on 10 SDS–polyacrylamide gel (SDS–PAGE) and transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% milk in TBST (50 mM Tris–HCl, pH 8; 154 mM NaCl and 0.1% Tween 20) for 2 h at room temperature, PVDF membranes were incubated overnight at 4°C with primaries antibodies against SIRT1 (1:1000, ab110304, Abcam) and actin (1:5000, MAB1501, Millipore). Membranes were incubated with the respective alkaline phosphatase conjugated secondary antibody, anti-mouse IgG (1:2500), HRP-linked antibody for 1 h at room temperature. Membranes were incubated with the ECL detection substrate (from Bio-Rad) and imaged with the Biospectrum Multispectral imaging system (UVP; LLC Upland, CA; Cambridge, UK). Densities of each band were calculated with ImageJ 1.45S program.

Docking study
The 3D structures of RSV and RSV-GS were drawn using HyperChem 7.5 (Froimowitz 1993), being minimized by the semi-empirical Polak–Ribiere conjugate gradient method (RMS<0.1 kcal/Å mol) (Zhang et al. 2006). Docking simulations between SIRT1 with bound peptide
substrate (pdb code: 5BTR) (Cao et al. 2015) and the small molecules were undertaken in AutoDock Vina (Scripps Research Institute, USA) (Seeliger & de Groot 2010, Trott & Olson 2010). AutoDock Vina considered the target conformation as a rigid unit while the ligands were allowed to be flexible and adaptable to the target. Vina searched for the lowest binding affinity conformations and returned 9 different conformations for each ligand. AutoDock Vina was run using an exhaustiveness of 8 and a grid box with the dimensions of 17.0, 19.0 and 17.0 Å, engulfing the crystallographic RSV-binding site. To validate the docking approach for the protein structure used, the root mean square distance (rmsd) between co-crystallized modulator RSV and docked RSV was evaluated. Conformations and interactions were visualized using PyMOL, version 1.3 (Lill & Danielson 2011).

Molecular dynamics simulation

Previous to the molecular dynamics (MD) simulations, the SIRT1:peptide:RSV-G and SIRT1:peptide:RSV complexes obtained from docking were placed inside a 6 Å margin spherical droplet containing 17234 water molecules and their energy minimized using the MMFF94x force field (Cheng et al. 2000) until RMS gradient <0.1 kcal/Å/mol. The MD simulations (Hospital et al. 2015, Mortier et al. 2015) were performed using MOE-dynamic implemented in MOE 2014.09 (Chemical Computing Groups, Montreal, Canada) (Vilar et al. 2008). MD simulations were done by choosing MMFF94x force field and NVT (N, total atom; V, volume; T, temperature) ensemble and Nosé-Poincaré–Andersen (NPA) algorithm (Sturgeon & Laird 2000), with 0.002 ps time step and sampling every 0.5 ps. The system was heated from 0 to 300 K in 100 ps (heat stage), followed by a 2000 ps of a production stage at 300 K; the system was then cooled back to 0 K in 100 ps (cooling stage) (Kapur & McCammon 2002). The system behavior was monitored by the analysis of root mean square deviation (RMSD) and potential energy over the course of the simulation.

Statistical analysis

Statistical evaluation of cytotoxicity results was carried out using one-way analysis of variance (ANOVA) followed by the Dunnett post hoc test. Statistical analyses were performed using the SPSS software (v 23.0; IBM). Statistical analysis of data involving studies of cell protection by the different molecules and Western blotting was performed by using GraphPad Prism 6.01 (GraphPad Software) and presented as mean ± standard deviation (s.d.) for the number of experiments indicated in the legends of the figures. Data normality was tested with Kolmogorov–Smirnov and Shapiro–Wilk test. Statistical analysis of two groups was performed using the non-parametric Mann–Whitney test or two-way ANOVA followed by Sidak post hoc test. Values with P<0.05 were considered as statistically significant (*).

Results

Synthesis and solubility of RSV-GS

The synthesis of RSV-GS was accomplished by sulfation of RSV-G with triethylamine sulfur trioxide adduct (4 equiv/OH), in dimethylacetamide. Applying MW irradiation, the reaction time decreased to only 30 min and a significant increase of the yield was achieved (85%) when compared to the previously described procedure in which conventional heating was used (Correia-da-Silva et al. 2011). Also, the use of SPE-WCX in the purification process contributed to improve the isolated yield of RSV-GS. Solubility of RSV-GS in water was found to be >1000 mg/mL (Fig. 1) and, accordingly to the European Pharmacopoeia, RSV-GS can be considered ‘very soluble’. This synthetic derivative is more than 1000 times more soluble in water than RSV or RSV-G (<1 mg/mL; Fig. 1). Water solubility of RSV and RSV-G was also evaluated in the same conditions for comparison purposes, and the obtained values (Fig. 1) were similar to those previously reported (Park et al. 2012).

Cytotoxicity testing

To investigate the potential benefits of the water-soluble RSV-GS, different cytotoxicity assays in HaCaT cells were performed in the presence of RSV-GS as also as in the presence of its parent non-sulfated compounds, RSV-G and RSV. The results showed that RSV promoted a concentration-dependent decrease in cell viability. In general, a significant decrease in HaCaT viability occurred at 100 μM. In the NR reduction assay (Fig. 2), exposure to 25 μM of RSV caused a significant decrease.
in cell viability comparing to the negative control (Fig. 2B), but the cell viability was around 80%, which can be considered non-cytotoxic (Technical Committee ISO/TC 194, 2009). A previous reported study, performed in a primary keratinocyte line, showed that RSV did not induce significant cytotoxicity up to 50 μM (Wu et al. 2014), which is consistent with the majority of our results. IC50 values were around 400 μM for all the assays except for MTT (174.47 ± 11.18 μM), which was the most sensitive assay (Table 1). This assay was also more sensitive when performed in HaCaT cells (Mukherjee et al. 2012). Of notice, the cell density used to perform the MTT test in the present work was lower than the one used for the other cytotoxicity tests, in agreement with optimization studies that defined the optimal experimental conditions. Thus, it is not possible to exclude the hypothesis that the lower IC50 values obtained for the MTT assay might be because cells were exposed to higher levels of the compound. These remarks further reinforce the importance of using different in vitro cytotoxicity tests, which rely on different mechanisms of cell death. The cytotoxicity assays used herein assess the lysosomal integrity (NR uptake), cell membrane damage (trypan blue) and metabolic activity (MTT and AlamarBlue/resazurin reduction). Considering the RSV-G, the concentration range studied was limited by the maximum concentration of the solvent (DMSO) that could be used without affecting cell viability (1%).

Even that, it was possible to observe that RSV-G did not affect cell viability at the tested concentrations (25–500 μM). Interestingly, the sulfated derivative RSV-GS only induced a significant reduction of HaCaT viability (70%) in the MTT reduction assay when a very high concentration was used (5000 μM) (Fig. 2A). A minimum tenfold increase in IC50 value can be expected for RSV-GS in comparison with RSV. These results are in accordance with previous data reported for sulfated small molecules in which no cytotoxicity was observed even at the millimolar range, namely in HCT-116 and SW-480 human colon cancer cell lines, human genital ME180, HeLa and primary human foreskin fibroblast cells, mouse lymphocytic leukemia P-388 cancer cell lines, human MT-4, MCF-7 and KB cells (Correia-da-Silva et al. 2014).

Figure 2
Cytotoxic effect of RSV and derivatives in HaCaT cells. Cell viability of HaCaT cell line exposed to RSV and derivatives, determined by the MTT reduction assay (A), neutral red uptake assay (B), Alamar Blue reduction assay (C) and trypan blue exclusion assay (D). Control 1 = cells treated with RSV and RSV-G solvent (DMSO): (A) 100 ± 13.68, (B) 100 ± 17.52, (C) 100 ± 29.13 and Control 2 = cells treated with cell medium: (A) 100 ± 16.16, (B) 100 ± 14.26, (C) 100 ± 11.57, (D) 100 ± 0.44. Each data point represents the mean ± S.D. of at least three independent experiments. *P ≤ 0.05 vs control (no additions).
Table 1  *IC*₅₀ values (mean±s.d.) and the values of the lowest concentration that promote a statistically significant decrease of HaCaT viability in comparison with control for different cytotoxicity assays.

<table>
<thead>
<tr>
<th>Cytotoxicity assays</th>
<th>MTT reduction</th>
<th>AlamarBlue reduction</th>
<th>Neutral Red uptake</th>
<th>Trypan Blue exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀±s.d.</td>
<td>Conc’</td>
<td>IC₅₀±s.d.</td>
<td>Conc’</td>
</tr>
<tr>
<td>RSV (µM)</td>
<td>174.47±11.18</td>
<td>100</td>
<td>387.27±6.88</td>
<td>350</td>
</tr>
<tr>
<td>RSV-GS (µM)</td>
<td>&gt;5000</td>
<td>5000</td>
<td>&gt;5000</td>
<td>–</td>
</tr>
<tr>
<td>RSV-G (µM)</td>
<td>&gt;500</td>
<td>–</td>
<td>&gt;500</td>
<td>–</td>
</tr>
</tbody>
</table>

(*IC₅₀*) concentration that promotes a reduction of 50% in cell viability.

*Conc*, Concentration at which a statistically significant (P ≤ 0.05) decrease in cell viability in comparison with control occurs

Protective effects on models of oxidative stress-induced cytotoxicity

Protective effects of RSV through several mechanisms of action have been demonstrated extensively in several disease models (Fukui et al. 2010, Albuquerque et al. 2015). To evaluate whether RSV-GS prevented H₂O₂- and menadione-induced cytotoxicity, metabolic activity and ATP levels were evaluated. Hydrogen peroxide and menadione are frequently used as cell stressors since they induce reactive oxygen species (ROS) formation, oxidative DNA damage, mitochondrial dysfunction and trigger apoptotic pathway, often resulting in an all-or-nothing result (Criddle et al. 2006, Loor et al. 2010, Reis et al. 2015, Xiang et al. 2016). Firstly, dose–response curves of H₂O₂ (9 mM) and menadione (25 µM) were obtained to choose cytotoxic concentrations for HaCaT cells (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Because the range of concentrations used did not allow for an accurate IC₅₀ determination and considering the large drop of ATP concentrations (often higher than 80%) after stressor incubation, we opted to use concentrations of stressors around the IC₅₀ based on metabolic viability results. In fact, the fact that ATP depletion may be an early consequence of different toxicants has been previously demonstrated (Swiss et al. 2013). Resveratrol per se decreased metabolic activity (Fig. 3A and B) and ATP levels (Fig. 4A and B) starting at 350 µM. Also, RSV did not prevent the decrease in metabolic activity resulting from H₂O₂ (Fig. 3A) and menadione (Fig. 3B) treatment, for all concentrations tested, while RSV increased ATP levels after H₂O₂ treatment at 25 and 50 µM (Fig. 4A). RSV-G per se had no effect in the metabolic activity (Fig. 3C and D) and ATP levels (Fig. 4C and D). However, RSV-G protected against the decrease on HaCaT metabolic activity after H₂O₂ treatment at 25, 50, and 100 µM (Fig. 3C) and protected from menadione-induced decrease in metabolic activity at 25 µM (Fig. 3D). In contrast, ATP depletion was not prevented after both treatments (Fig. 4C and D). Regarding RSV-GS per se, 100, 350 and 500 µM decreased metabolic activity (Fig. 3E and F), while no alterations in ATP levels were observed, with exception of increased ATP levels after pre-treatment with 5000 µM RSV-GS (Fig. 4E and F). Moreover, RSV-GS did not protect from H₂O₂ and menadione-induced decrease metabolic activity (Fig. 3E and F) and ATP depletion (Fig. 4E and F). The results obtained using resazurin in the presence of menadione as a stressor must be analyzed carefully. By obtaining the absorption spectra of resazurin in the presence of menadione, a slight increase in the spectrum of the former was observed. Therefore, it was not possible to conclude whether this type of interaction also occurs inside cells during the experimental protocol (Supplementary Fig. 2).

Western blotting, docking and MD studies

Sirtuins are attractive therapeutic targets for metabolic and aging-related diseases (Lavu et al. 2008, Shih & Donmez 2013). Sirtuins employ a conserved catalytic core domain to catalyze deacetylation by transferring the acetyl group from the acetyl-lysine of proteins to NAD⁺ (Sauve 2010). Sirtuins activating compounds, such as RSV-related polyphenols can promote survival of human cells. However, their mode of action is still not completely understood (Chung et al. 2010, Jayasena et al. 2013).

Initially, SIRT1 protein content was semi-quantified using Western blotting after treatment with RSV and derivatives. The obtained results (Fig. 5) showed that RSV and RSV-GS increased SIRT1 protein content in HaCaT cells at 100 and 500 µM, respectively, while no alterations were observed after RSV-G treatment. Moreover, in order to predict the molecular mechanism of RSV-GS, an in silico docking study was performed between the small molecules, RSV-GS and the known activator RSV and the target SIRT1. As AutoDockVina was able to return a RSV pose below a preselected RMSD value from the known conformation (bellow 2 Å), it was assumed that it could predict docking poses accurately. The RMSD value found...
for crystallographic RSV vs docked RSV was 0.3 Å, which was considered a good threshold value for validating a structure for use in molecular docking (Fig. 6C) (Hevener et al. 2009). Both RSV and RSV-GS bind on top of the substrate peptide (Fig. 5A); they bind in close proximity and directly contact each other through π-π stacking interactions and polar contacts. It has been described that peptide leads to the formation of the activator-binding site by influencing these regions (Gertz et al. 2012). The RSV-GS:peptide:SIRT1 and RSV:peptide:SIRT1 complexes obtained on the docking studies presented a free energy of −9.1 and −8.2 kcal/mol, respectively. Therefore, RSV-GS binds more stably to SIRT1 (lower binding free energy) than the parent compound, RSV. The 6 sulfate groups of RSV-GS increased the number of polar interactions with SIRT1-binding pocket (interactions with residues Pro-211, Thr-219, Gln-222, Ile-223, Asn-226, G1u-230, Asp-298 and Phe-414) (Fig. 5C). Both compounds RSV and RSV-GS adopted a similar binding pose in SIRT1-binding pocket, with the phenol groups establishing interactions with the sub-pocket formed by residues Pro-211 and Asn-226 (Dai et al. 2015). However, RSV-GS adopted a pose that also reached the surfaces formed by residues Asp-298 and Glu-230 (Fig. 5B). Similar to RSV (Gertz et al. 2012), RSV-GS shall close SIRT1 active site opening, thereby trapping the bound peptide and increasing the interaction interface by contacting the substrate fluorophore directly. This interaction may lead to a substrate binding mode more suitable for the subsequent deacetylation step (Cao et al. 2015).

To gain detailed insight in the energetic and geometric behavior of the SIRT1:peptide:RSV-GS complex in aqueous solution, a 2 ns MD simulation was performed based on the aforementioned docking complex structure considering the effects of the target flexibility and the explicit water solvation. Conformations of SIRT1:peptide:RSV-GS complex along simulation time (Fig. 7A, B, C and D), RMSD and potential energy plots of complex conformations with respect to time (Fig. 7E) were obtained.

During the simulation period, the RSV-GS and peptide positions on the binding pocket was kept constant, with slight variations in the conformation, as expected. Water molecules established hydrogen interactions with polar groups on both RSV-GS and peptide (Fig. 7A, B, C and D). By calculating the RMSD values of the RSV-GS backbone, heavy atoms relative to the coordinates of the initial (energy-minimized) structure, the overall changes in RSV-GS atomic coordinates were monitored during MD simulations in water (Fig. 6E). After an initial fast-rise region, the RMSD values changed little, reaching a more constant value after approximately 1 ns of simulation.
A sulfated resveratrol derivative

M Correia-da-Silva, V Rocha et al.

M35

Discussion

The physicochemical properties of RSV have restricted its use in topical products. One particular challenge is the poor water solubility, as confirmed in this work (0.029 ± 0.005 mg/mL), that prevents the use of high concentrations of pure RSV into topical formulations. In fact, the leading topical RSV products often contain less than 1% of pure RSV (Farris et al. 2013). The synthetic persulfated resveratrol glucoside described in this work was found to be around 1000-fold more soluble in water than RSV. Topical formulations comprise a wide array of physical systems being oil in water creams one of the most used, for which water is the external phase and the prominent ingredient in their composition. This favors the inclusion of water-soluble compounds in meaningful concentrations regarding biological activity and a hydrophilic RSV derivative represents a clear advantage over the parent molecule. The improvement in RSV penetration has been studied with several approaches, including formulation design and the use of pro-molecules (Zhang et al. 2007, Hung et al. 2008). Enhancement of RSV bioavailability in the epidermis upon application of a water-soluble pro-molecule has been reported (Zhang et al. 2007).

Toxicological and protective testing in a human keratinocyte cell line is a logical approach for compounds intended for skin care application. Remarkable differences were observed between the cytotoxic effects of RSV-GS and RSV. Resveratrol decreased the cell viability more than 50% at 500 µM while RSV-GS did not decrease the cell viability for more than 30% even at 5000 µM. However, the non-sulfated RSV-G was also much less toxic than RSV.

Figure 4

Effects of RSV and derivatives in ATP levels of HaCaT cells in oxidative stress models. Cells were incubated with 25, 50, 100, 350 and 350µM RSV or RSV-G or with 100, 350, 500, 1000 and 5000µM RSV-GS for 24h, followed by incubation with hydrogen peroxide (H₂O₂) and menadione for 3h. Intracellular ATP levels of HaCaT cells incubated with RSV (A and B), RSV-G (C and D) and RSV-GS (E and F) were obtained by CellTiter-Glo Luminescent Cell Viability Assay, respectively, as described in materials and methods section. Data are expressed as mean ± s.d. of 4 independent experiments. For white bars, ****stands for P<0.0001 vs control. For black bars, $$$stands for P<0.0001 vs control. For the comparison between the two groups, ####stands for P<0.0001 vs control group.

Figure 5

RSV and RSV-GS increased SIRT1 protein content. Cells were incubated with 25 and 100 µM RSV or RSV-G and with 100 and 500 µM RSV-GS for 24h. Basal sirtuin 1 content after RSV or derivatives treatment was semi-quantified by Western blotting analysis (A and B). Blots are representative from four different cell preparations. Data are expressed as mean ± s.d. of 4 different experiments. *P<0.05 denotes a significant difference from control.
(in contrast to RSV, no decrease in cell viability was observed in the presence of 500 µM of RSV-G). It is possible to argue that, while glycosylation had guaranteed the non-toxicity of RSV, sulfation guaranteed both water solubility and non-toxicity. Besides water solubility, this adds another advantage to RSV-GS: the possibility of use RSV-GS in higher concentrations on topical products than RSV.

Some similarities between the protective effects of RSV-GS and RSV against well-characterized oxidative stress inducers were observed. Interestingly enough, RSV-GS and RSV (the positive control) did not prevent H$_2$O$_2$- and menadione-induced decrease metabolic activity; however, RSV at 25 and 50 µM protected from ATP depletion induced by H$_2$O$_2$, while RSV-GS did not. Despite some authors have shown in cells the protective effects of RSV against H$_2$O$_2$-induced toxicity (Ovesna et al. 2006), some authors also described the opposite effect (Fabre et al. 2011), anticipating that RSV and RSV-GS effects are cell type and dose dependent and that studies involving different variable stressors and incubation times make different studies hard to compare. Furthermore, it was also described that RSV at 50 µM increased the susceptibility to stressors (Fabre et al. 2011); hence, the use of resveratrol as a positive control does not always represent a universal assumption. This is in accordance with the results obtained, where RSV, but not RSV-GS, increased menadione-induced toxicity. Nevertheless, it is known that RSV induces cell cycle arrest and that cells in S-phase are significantly more susceptible to H$_2$O$_2$ than in other phases of the cell cycle (Leroy et al. 2001, Frankenbergschwager et al. 2008), which can explain the obtained results. Moreover, the increase in ATP levels observed after 5 mM RSV-GS might be a metabolic adaptive response, which precedes programmed cell death (Los et al. 2002).

Nevertheless, we also demonstrated here that RSV and RSV-GS increased SIRT1 protein content, which is in agreement with others reports (Price et al. 2012, Deus et al. 2017). Sirtuin-activating compounds, such as RSV-related polyphenols, can promote survival of human cells, although their mechanism is not completely understood (Chung et al. 2010, Jayasena et al. 2013). To clarify whether the studied molecules directly interact with SIRT1, in silico studies in this work have predicted that RSV-GS establishes polar interactions with eight SIRT1 residues,
whereas RSV establishes only two polar interactions. At this point, the extent of hydrolysis by sulfatases in the skin is uncertain. However, even if biotransformation of RSV-GS partially occurs, the effectiveness is not expected to be compromised since binding to SIRT1 was predicted to be more efficient and high concentrations of RSV-GS will be possible to incorporate on topical products.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JME-18-0031.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported through national funds provided by Foundation for Science and Technology from the Minister of Science, Technology and Higher Education (FCT/MCTES – PIDDAC) and European Regional Development Fund (ERDF) through the COMPETE – Programa Operacional Factores de Competitividade (POFC) (POCI-01-0145-FEDER-016597/007440, POCI-01-0145-FEDER-016790 and POCI-01-0145-FEDER-016793), PPCDT Factores de Competitividade (POFC) (POCI-01-0145-FEDER-016659/007440, and Higher Education (FCT/MCTES – PIDDAC) and European Regional Development Fund (ERDF) through the COMPETE – Programa Operacional Factores de Competitividade (POFC) (POCI-01-0145-FEDER-016597/007440, POCI-01-0145-FEDER-016790 and POCI-01-0145-FEDER-016793), PPCDT and Technology from the Minister of Science, Technology and JNK in cultured skin keratinocytes. Journal of Cellular and Molecular Medicine 13 3632–3643. (https://doi.org/10.10111/j.1582-4934.2008.00453.x)

Author contribution statement
M C S synthesized the compound and determined its physicochemical properties and E S and M P analyzed the data. V R performed the cytotoxicity analysis with HaCaT cells and I A and C M analyzed the data. V R performed the in silico studies. C M D, A M C and P J O performed measurements of protective effects of RSV and derivatives against stressors and Western blotting analysis. M C S, V R, and A P wrote the manuscript, while all authors gave significant contributions in discussion and revision. E S and I A conceived the study design.

References


Kasiotsis KM, Pratsinis H, Kletas D & Haroutoumania SA 2013 Resveratrol and related stilbenes: their anti-aging and anti-angiogenic properties. *Food and Chemical Toxicology* 61 112–120. (https://doi.org/10.1016/j.fct.2013.03.026)


Rocha V, Marques C, Figueiredo JL, Gaião AR, Costa PC, Sousa Lobo JM & Almeida IF 2017 In vitro cytotoxicity evaluation of resveratrol-loaded nanoparticles: focus on the challenges of in vitro...
methodologies. *Food and Chemical Toxicology* **103** 214–222. (https://doi.org/10.1016/j.fct.2017.03.017)


Received in final form 14 March 2018
Accepted 27 March 2018