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ORIGINAL ARTICLES

The Cell Membrane is the Main Target of Resveratrol as Shown by Interdisciplinary Biomolecular/Cellular and Biophysical Approaches

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Abstract One of the research lines developed in our laboratory is focused on the study of the bioactivity of natural substances. Resveratrol (RV) is a polyphenol non-flavonoid compound present in a number of plant species but mainly in the berries of the red grape *Vitis vinifera*. The powerful antioxidant action of this molecule is well documented. In this work we evaluated the effects of this substance by adopting diverse experimental strategies. In particular, we studied the effects on cell vitality and cycle by MTT and cytofluorimetric assays. In addition, we explored the action of RV on the cell membrane by a well-consolidated biophysical approach: electrorotation. This technique allows assessment of the structure/function of the cell membrane. The results presented here demonstrate that RV shows a modest effect on the biological properties of the cell in terms of cytotoxicity and cell cycle alterations. On the contrary, a significant effect on the membrane structure/function was observed, consisting of an enhanced intramembrane ion transport. The implications and interpretation of these membrane alterations are discussed.

Keywords Resveratrol · Cytotoxicity · Cell membrane · Cytofluorometry · Electrorotation

Introduction

Resveratrol (RV) is a polyphenol nonflavonoid compound belonging to the family of natural phytoalexins which are produced by plants to combat the attack of pathogens and/or stress. This molecule is particularly abundant in the berries of the red grape (*Vitis vinifera*) but is also present in highly pigmented vegetables and fruit such as pumpkin (*Curcuma longa*) or black mulberry (*Morus nigra*) as well as peanuts and pine kernels. The desiccated roots of the Japanese knotweed (*Polygonum cuspidatum*) are particularly rich in RV since they contain a 400-fold higher amount of the drug compared to red grapes. The use of RV in traditional Oriental medicine dates back several centuries. The drug shows a great number of diverse biological activities, such as antitumor, antioxidant, phytoestrogen and antiviral activities, just to mention a few.

Its antitumor properties were ascribed to the ability of the drug to modulate negatively cyclin-cdk-dependent cell mechanisms. Cells exposed to RV undergo an arrest in G_1 phase followed by apoptosis; this phenomenon was first reported in human epidermoid carcinoma (Ahmad et al. 2001). Its powerful antioxidant character has been demonstrated in several different conditions such as chronic obstructive pulmonary disease and microbial infections (Hosoda et al. 2012; Biswas et al. 2013; Li et al. 2012; Ding et al. 2012). Due to its antioxidant properties, RV decreases platelet aggregation; therefore, this drug may find a clinical role in the control of the formation of atherogenic plaques (Olas et al. 1999).

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Phytoestrogens are nonsteroid molecules produced by plants that exert their effects mainly by binding to specific estrogen receptors (ERs) (Turner et al. 2007). These have different biological actions, among which is an antitumor action. RV has a structural resemblance to phytoestrogens, and it has been suggested that it may interact with the ER, thus implying a possible phytoestrogenic function. As matter of fact, it has been shown that at low concentration RV partially antagonizes the proliferative action of 17β-estradiol (Lu and Serrero 1999).

The antiviral action of RV has also been demonstrated in herpes simplex virus, where it inhibited expression of the viral genes and the production of viral DNA (Faith et al. 2006). The completion of the productive infection of influenza A and varicella zoster viruses seems to be inhibited by RV in cultured cells and in a mouse model of vaginal infection (Palamara et al. 2005; Docherty et al. 2006). Analogous properties of RV against herpes virus simplex were shown also in animal models. However, in this latter case, suppression of the transcription factor NF-κB seems to be involved in its antiviral property (Faith et al. 2006; Docherty et al. 2005). Recent evidence exists that RV may exert an antiviral action against HIV and Epstein-Barr virus (Clouser et al. 2012; De Leo et al. 2012; Campagna and Rivas 2010).

In our laboratory we demonstrated that RV inhibits the DNA replication of murine polyomavirus in mouse fibroblasts in culture, possibly acting during the phase of viral penetration. Exposure to the drug was carried out at a concentration of RV which did not show a significant cytotoxic effect. At higher doses the drug shows a cytotoxic effect, but this is only apparently paradoxical; as a matter of fact this drug induces cell cycle arrest and stimulates the mitochondrial pathway activated by reactive oxygen species, thus leading to apoptosis (Saiko et al. 2008; Juan et al. 2008). An analogous action has been described for another potent antioxidant, curcumin, which is able to induce apoptosis in human cervical cancer cells (Singh and Singh 2009). In any case, it is known that RV can exert antioxidant and anti-inflammatory activities; but, in addition, it regulates multiple cellular events associated with carcinogenesis (for a relatively recent review, see Delmas et al. 2006).

In this work, the biological effects of the interaction of RV with cells were investigated by biomolecular/cellular and biophysical strategies. We evaluated the extent of cytotoxicity, the kinetics of uptake and the effects on cell cycle progression caused by RV. A noninvasive biophysical strategy, based on electrorotation, was already used to study the membrane behavior of prokaryotic and eukaryotic cells subjected to chemical and biological stresses (Gimsa et al. 1989, 1991; Dalton et al. 2004; Graça da Silveira et al. 2002; Lee et al. 2010; Berardi et al. 2009a). In particular, our work suggested that RV may cause damage of the plasma membrane (Berardi et al. 2009b). With respect to this, electrorotation allows monitoring of the alterations of the plasma membrane induced by a variety of external stimuli. Measurement of the membrane dielectric parameters C and G (capacitance and conductance per unit area, respectively) provides effective information on the membrane structure/function mainly on ion transport.

Materials and Methods

Cell Cultures

The mouse fibroblast line 3T6 was used throughout the work. Cells were grown in high-glucose DMEM, supplemented with fetal calf serum (10 % final concentration), glutamine (50 mM) and penicillin–streptomycin (10,000 U/ml). Growth temperature was 37 °C in controlled humidity at 5 % CO_2. Cells were routinely subcultured every third day and treated with RV at final concentrations of 50 and 100 μM for 24 or 48 h if not otherwise specified. In all experiments, the samples contained 0.02 % DMSO in PBS, which represents the final concentration in which the drug was dissolved. Treatments started 24 h after plating.

Assessment of RV Cytotoxicity

Cell viability was evaluated by the quantitative colorimetric Mosmann assay (Mosmann 1983). The method is based on the use of 3-(4,5-dimetiltiazol-2-yl)-2,5-difenil tetrazolium-bromide (MTT), a yellow, water-soluble salt. A vitality deficit is evidenced when MTT is converted into insoluble purple salts, whose absorbance, measured at 570 nm, can be directly converted into the number of vital cells.

Absorption Kinetics

The kinetics of RV uptake by living 3T6 cells was assessed after incubation with 100 μM RV at different times (2, 10 and 60 min). Samples were trypsinized, washed with PBS, stained with 40 μg/ml propidium iodide (PI) and immediately analyzed. Fluorescence signals were evaluated with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 15-mW, 488-nm and air-cooled argon ion laser. PI fluorescence emission was collected through a 575-nm band pass filter and acquired in "log" mode.

Cell Cycle

Cells grown to semiconfluence were treated with RV, and both floating and adherent cells were collected, washed twice with cold PBS and centrifuged. The pellet was fixed in 70 % ethanol/H_2O at 4 °C for 1 h, washed twice with cold PBS and resuspended in the same buffer containing 40 μg/ml PI and 100 μg/ml RNase. Samples were incubated at 37 °C for 1 h and then analyzed on the FACScan flow cytometer. At least 10,000 events/sample were acquired in linear mode. The percentage of cells in the different phases was calculated using CellQuest software (Becton Dickinson) (Calcabrini et al. 2006).

Assessment of Apoptotic Cells

To detect phosphatidylserine (PS) translocation, an annexin V-FITC kit (MBL, Nagoya, Japan) was used. Cells were treated with 100 μM RV for 24 and 48 h at 37 °C. After treatment, cells were centrifuged and resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.5), 140 mM NaCl and 2.5 mM $CaCl_2$]. Suspensions were incubated with 1 μg/ml of annexin V-FITC and with 1 μg/ml PI for 10 min at room temperature in the dark. Populations of annexin V-positive/PI-negative cells (early apoptosis) and annexin V-positive/PI-positive cells (late apoptosis) were evaluated by flow cytometry. Both floating and adherent cells were collected, labeled for 10 min at room temperature with annexin V-FITC conjugate (1 μg/ml, final concentration) and with PI (40 μg/ml, final concentration) and immediately analyzed on a flow cytometer (Juan et al. 2008).

Electrorotation: Theory and Apparatus

A rotating electric field, applied to a polydispersed cell suspension, induces a dipole moment due to the accumulation of charges at the interphase plasma membrane/solvent. This charge accumulation is a consequence of the high difference in polarizability between the dispersion medium and the cell membrane. When this mechanism of interfacial polarization relaxes, a phase shift between the electric field and the induced dipole moment occurs. As a consequence, a torque moment is generated. The rotation period of the cell depends upon the frequency according to the Debye model (Hasted 1973). The frequency of relaxation changes with the conductivity of the dispersing medium. A linear fit of the relaxation frequency, measured at different solvent conductivities, allows calculation of the membrane parameters' specific capacitance and conductance (C and G, respectively). An exhaustive overview of the electrorotation theory can be found in the literature (Arnold and Zimmermann 1982, 1988; Gimsa et al. 1991; Gimsa 2001, see also Supplementary Data). It is worth noting that in nonliving cells the dielectric insulating property of the membrane vanishes; therefore, the cells do not rotate. Therefore, this technique takes into account only metabolically active cells. We used a standard apparatus where the rotating electrical field was generated by superimposing four square waves out of phase by 90° with respect to each other. The square pulses were applied to the copper miniplate electrodes of the measuring chamber, which forms a central circular cavity with a volume of about 10 μl. The whole setup was glued onto a microscope slide. The apparatus was connected to a video-recording system that permitted a more accurate offline image analysis. This analysis consisted of measurement of the cell radius and of the rotation period at each frequency of the electric field in four dispersion media with different conductivities. These media consisted of an osmolar sucrose solution (300 mM) supplemented with NaCl at concentrations of 0, 0.5, 1.0 and 1.5 mM. At minimum, 15 cells were observed. The conductivities of the four solvents were accurately measured by an automatic impedance meter (HP 4194A; Hewlett-Packard, Palo Alto, CA, USA). The experimental setup was previously described in detail (Cosimati et al. 2013).

Statistical Analysis

All experiments were independently repeated three times. Statistical analysis was done by a one-way ANOVA, followed by a comparative least significant difference test. Results were considered significant at $p < 0.05$.

Results and Discussion

Cytotoxicity Evaluation of RV

In the first series of experiments we assessed the cytotoxicity of RV by two different methods. The first one is the well-known MTT test, which is based on the reduction of tetrazolium salts operated by the mitochondrial enzyme succinate dehydrogenase. This reaction occurs only in metabolically active cells and produces blue formazan crystals that can be spectrophotometrically measured. The absorbance values thus obtained are directly converted to the actual number of vital cells. Figure 1 (upper panel) shows that RV did not cause a relevant effect after 24 h of treatment at all concentrations, although at 100 μM about 80 % of the cell population was not affected by the treatment. After 48 h of treatment, however, the population of proliferating cells was about 60 % compared to controls (Fig. 1, lower panel). We assessed cell viability also by the "classical" vital dye exclusion with trypan blue. The cell

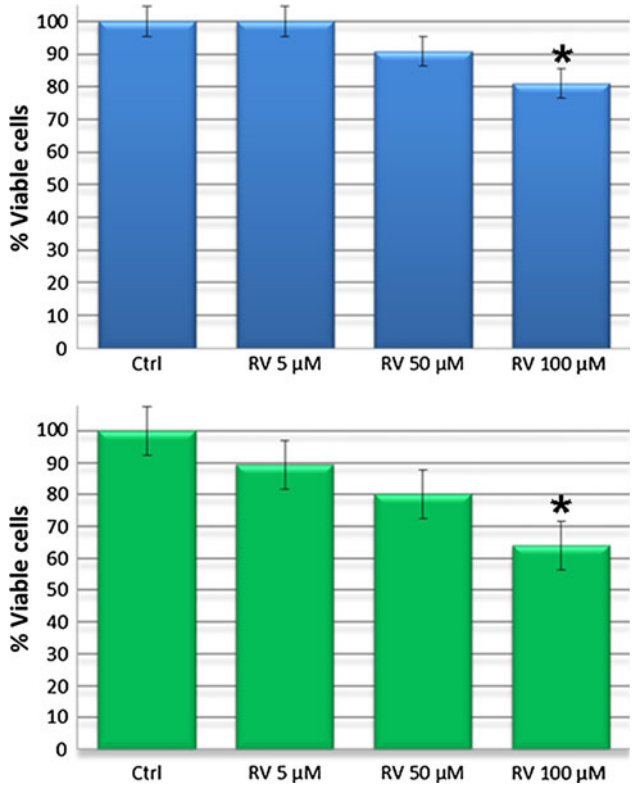

Fig. 1 Assessment of cell proliferation/vitality by the MTT assay. *Bars* report the rate of cell survival after 24 or 48 h of treatment (*upper* and *lower panels*, respectively) as measured by three independent MTT assays (one-way ANOVA $*p < 0.01$). *RV* resveratrol

Table 1 Percentage of dead cells after vital dye exclusion

Treatment time (h)	Control (0 µM)	5 µM	50 µM	100 µM
24	6 ± 1	8 ± 1	22 ± 1	30 ± 2
48	11 ± 1	20 ± 1	32 ± 2	45 ± 2

The table reports the percentage of dead cells after treatment with trypan blue for the indicated times. The percentage refers to a total count in the order of 200 cells. Values were obtained by three different independent experiments

count was in agreement with the data obtained by the MTT assay (Table 1). One should take into account, however, the intrinsic difference between the two techniques. The vital dye exclusion analyzes more general aspects of cell vitality such as the activity of the mechanisms of transport across the membrane. On the contrary, the MTT assay is specifically informative for mitochondrial activity.

Plasma Membrane Integrity Evaluation After RV Treatment and Cell Cycle Analysis

In the light of the results reported above, to obtain information about the alteration of the plasma membrane

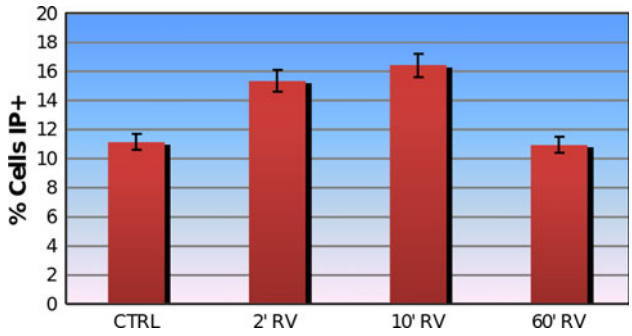

Fig. 2 Kinetics of RV intracellular absorption. PI staining of cells measured by FACS analysis after interaction with 100 µM RV (2, 10 and 60 min, respectively). Results are expressed as the mean percentage of PI-stained cells ± SD from three independent experiments. *RV* resveratrol, *CTRL* control

permeability induced by RV exposure, we performed an uptake kinetic experiment by flow cytometry to evaluate the PI entry into the cells. PI is a vital dye normally excluded from cells. If the cell membrane integrity has been compromised, PI freely crosses the membrane. Data in Fig. 2 show the percentage of PI-positive cells, which provides an indirect evaluation of membrane permeability. The absorption kinetic was carried out in a short time, 2–60 min after treatment with RV. The results indicate that a change in membrane permeability occurs mainly in the first 10 min. The functionality of the membrane seems to be restored after 60 min. This suggests that the phytoalexin interaction is very rapid, leading to a membrane permeability transient modification. Moreover, this result supports previous observations from our laboratory showing that RV causes alterations of plasma membrane permeability by electrorotation as well as classical biomolecular/cellular techniques (Berardi et al. 2009a, b).

The effect of treatment with RV on the progression of the cell cycle was also investigated by flow cytometry. In the literature it is reported that the drug is capable of inducing arrest in the G_1 phase of several cell lines but especially of different tumor lines (Ahmad et al. 2001; Notas et al. 2006; Bai et al. 2010; Soto et al. 2011). Incidentally, this specific biological activity makes the drug an important candidate for treatment of this disease, although this remains a matter of debate (Vang et al. 2011).

In any case, flow-cytometric analysis showed that RV treatment did not exert a significant influence on the cell cycle (Fig. 3). The same percentage of cells was present in control and treated samples in the various phases of the cycle. RV treatments for 24 h did not seem to induce arrest in G_1 phase, while in different tumor cell lines the drug caused the opposite effect. Since RV is able to penetrate the plasma membrane, as shown by the MTT, trypan blue and PI results, the absence of a significant effect on the cell cycle is surprising. Similar behavior was observed also

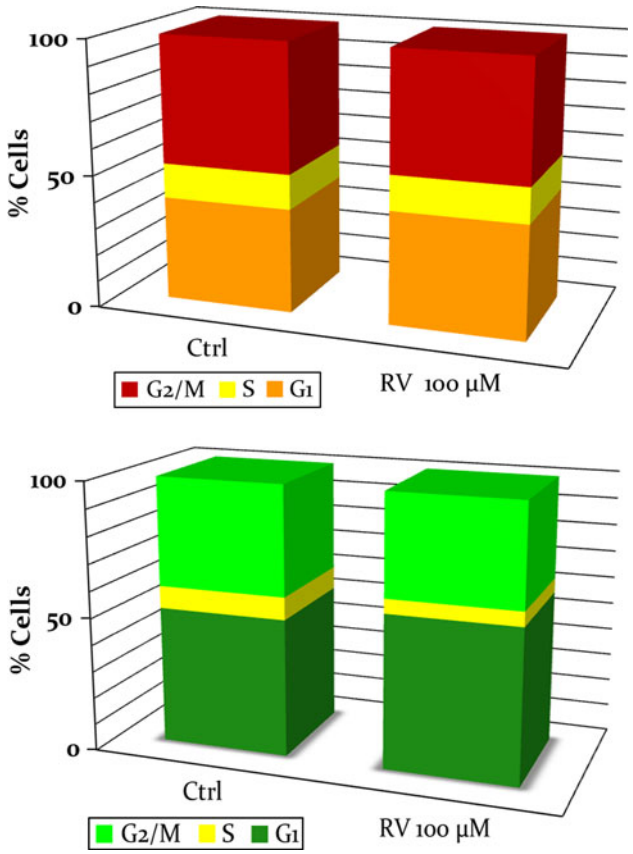

Fig. 3 Analysis of cell cycle by flow cytometry. Suspensions of 3T6 cells were treated for 24 or 48 h with 100 µM resveratrol (RV; *upper* and *lower panels*, respectively). Both floating and adherent cells were collected, processed for PI staining and analyzed on FACScan. *Different colors* indicate the percentage of cells found in the different phases of the cell cycle (G_1, S, G_2/M). Relative percentages of each subpopulation were calculated using CellQuest software (Becton Dickinson) and represented graphically. Data were obtained by three independent experiments, and the errors were between 5 and 8 %. *CTRL* control (Color figure online)

after 48 h of treatment. Compared to the 24-h results, however, there is an increase of the cell number in G_1 phase, associated with a limited decrease in S phase of about 10 %. It has also been reported that the arrest in the G_1 phase is related to activation of the apoptotic process (Ahmad et al. 2001). Since we observed cell death, although this phenomenon occurs at a rather limited level, we investigated whether this subpopulation of cells might undergo apoptotic death.

Exposure of PS on the Outer Cell Surface

We analyzed the level of apoptosis induced by RV through flow-cytometric analysis, evaluating the exposure on the outer membrane surface of PS, an early marker of apoptosis. As shown in Fig. 4, no appearance of apoptotic cells, defined as annexin V-positive/PI-negative, was observed. These 3T6 cells appeared to be more resistant to apoptosis than the other cell lines, even though RV was able to induce a minor block of the overall cell proliferation in G_1 phase. These results were also supported by the analysis of chromatin condensation and nuclei fragmentation after Hoechst labeling (data not shown). In any case apoptosis did not seem to occur at significant levels in the case of 3T6 cells exposed to RV in our experimental conditions. In conclusion, the results shown in Fig. 1 suggest a defective proliferation rather that an actual cell death phenomenon.

The Structure/Function of the Cell Membrane Investigated by Electrorotation

RV is promptly assumed by the cell, as shown by the PI kinetics. However, the entry of the drug across the membrane does not seem to have dramatic consequences on survival and on the cell cycle. Previous results obtained in our laboratory demonstrated that RV mainly influences the structure/function of the membrane, increasing its fluidity (Berardi et al. 2009b). On the basis of these results, we analyzed the action of the drug by electrorotation. This very powerful tool allows investigation of the biophysical properties of the plasma membrane at the single-cell level and in a noninvasive fashion. The technique measures two membrane parameters, specific capacitance (C) and conductance (G). The results of these measurements are reported in Table 2. It is evident that after 24 h of treatment no variation of the dielectric parameters was apparent. After 48-h treatment, however, a very small effect on C was observed and, on the contrary, an evident and large increase of G occurred. C is strongly linked to the structure and morphology of the membrane: on the basis of the results reported in Table 2, these do not undergo significant modifications. The large increase of G implies a dramatic effect on the overall membrane function in terms of an enhanced ion transport. The cytofluorimetric data show the presence, although negligible, of necrotic cells. In any case, these do not make any contribution to the variation of the membrane parameters since they do not rotate and therefore are not "visible" by this technique. Also, as mentioned above, we monitored a small fraction of apoptotic cells by cytofluorimetry. On the contrary, apoptotic cells do rotate up to a certain stage of the process (Bonincontro et al. 2007). However, due to their scant number, the contribution to the variation of the average values of the membrane parameters is irrelevant. In the case of other natural substances as well as supramolecular aggregates, a decrease of C and G was observed (Cosimati et al. 2013; Bonincontro et al. 2007). In conclusion, the maintenance of the C value strongly suggests that RV has no effect on membrane structure. However, as far as G is concerned, the RV-treated membrane shows the opposite effect, which can be

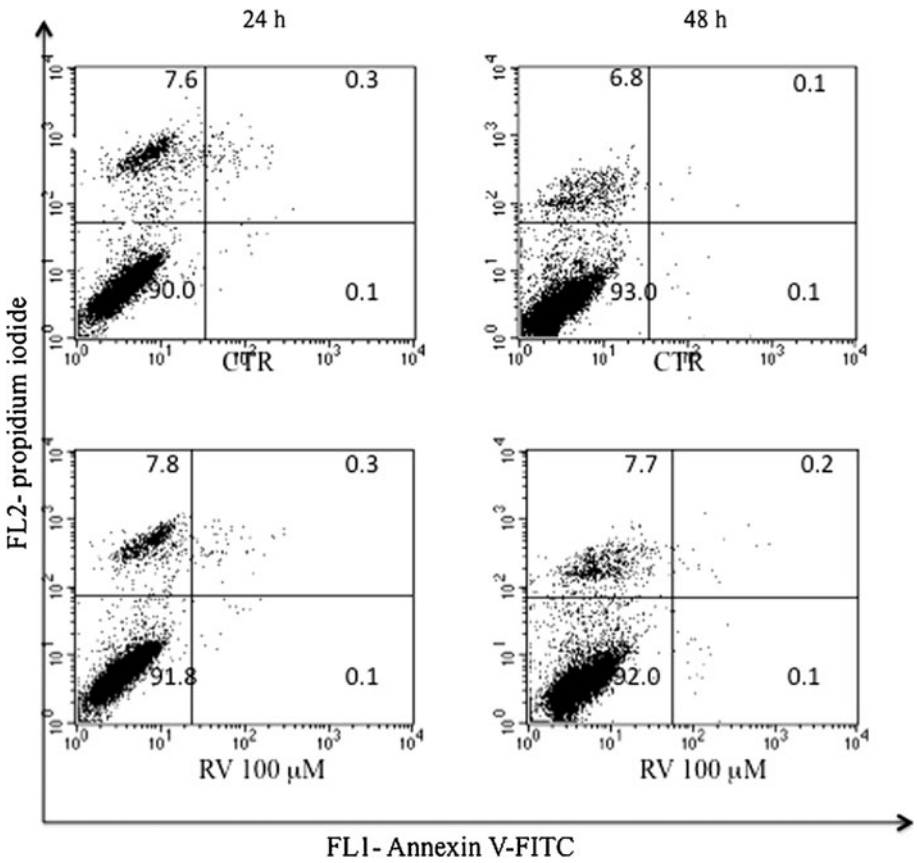

Fig. 4 Evaluation of apoptosis induced by RV. Cell surface annexin V binding was studied by flow cytometry. Cells were treated with 100 μM RV for 24 and 48 h (*right* and *left panels*, respectively). At both times of treatment floating and adherent cells were collected and labeled with conjugated annexin V-FITC (1 μg/ml). The percentage of apoptotic cells refers to annexin V-positive/PI-negative cells, while necrotic cells appear annexin V-negative/PI-positive. Values represent the mean ± SD of three independent experiments. *CTRL* control

Table 2 Effect of RV treatment on membrane dielectric parameters

	C (μF/cm^2)	G (S/cm^2)
Untreated cells (24 h)	0.9 ± 0.1	0.27 ± 0.03
RV-treated cells (24 h)	0.7 ± 0.1	0.26 ± 0.03
Untreated cells (48 h)	1.0 ± 0.1	0.29 ± 0.04
RV-treated cells (48 h)	1.3 ± 0.1	0.60 ± 0.06

Values of specific capacitance (C) and conductance (G) for untreated 3T6 control cells and RV-treated suspensions. The table reports the values at 100 μM concentration of drug

associated with enhanced cell metabolism as observed by other strategies and in wholly diverse cell models (Xu and Si 2012). Finally, it is commonly accepted that the anti-oxidant action of RV strongly reduces the formation of free radicals whose presence hinders ion transport, which may lead to cell death.

Conclusions

The cytotoxicity of RV was assessed by two different methods: MTT and vital dye exclusion (trypan blue and PI). The drug did not cause relevant mortality after 24 h of treatment at all concentrations. After 48 h of treatment, however, the number of proliferating cells decreased significantly. The absorption kinetics of the drug was indirectly determined by measuring the percentage of PI-positive cells. The overall result suggests that RV interaction with the cell membrane is very rapid and that this leads to its modified permeability. Flow-cytometric analysis showed that RV does not exert a significant influence on the cell cycle, although at longer treatment times a small subpopulation of cells arrested in G_1 was observed. In any case this does not seem to cause a relevant level of apoptosis as measured by the PS translocation to the outer surface of cell membrane. Electrorotation analyzes the biophysical parameters of the membrane (C and G) only of vital cells. The specific capacitance (C) is linked to the structure and morphology of the membrane and did not substantially vary in our measurements. On the contrary, the specific conductance (G), which is related to membrane function, showed an evident and large increase. This increase is related to enhanced ion transport after treatment with RV. It is known that the anti-oxidant action of RV reduces the formation of free radicals, thus hindering ion transport. Taken together the results presented in this work show that the main target of RV action is the cell membrane.

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