

# **Antiproliferative activities of resveratrol and related compounds in human hepatocyte derived HepG2 cells are associated with biochemical cell disturbance revealed by fluorescence analyses**

DIDIER COLIN<sup>1</sup>, ALLAN LANCON<sup>1</sup>, DOMINIQUE DELMAS<sup>1</sup>, GERARD LIZARD<sup>1</sup>, JESSICA ABROSSINOW<sup>1</sup>, EDMOND KAHN<sup>2</sup>, BRIGITTE JANNIN<sup>1</sup> & \*NORBERT LATRUFFE<sup>1</sup>

<sup>1</sup> INSERM U 866; Université de Bourgogne, Laboratoire de Biochimie Métabolique et Nutritionnelle, Dijon, France

<sup>2</sup> INSERM U 678; CHU Pitié-Salpêtrière, Paris, France

## **Abstract**

Resveratrol is a well known polyphenol largely produced in grapevine. It is a strong antioxidant and a free radical scavenger. It exhibits several beneficial effects for health including cancer. Resveratrol antioxidant activity is essential in the prevention of chemical-induced cancer by inhibiting initiation step of carcinogenesis process but it is also considered to inhibit cancer promotion and progression steps.

While the effects of resveratrol on cancer cells are widely described, the data available on the antiproliferative potential of resveratrol derivatives remain weak. Nevertheless, resveratrol analogues could exhibit stronger potentials than the parent molecule. So, we compared the cellular effects of *trans*-resveratrol, *trans*- $\epsilon$ -viniferin and their respective acetate derivatives, as well as a polyphenol mixture extracted from grapevine shoots, called vineatrol. We studied their abilities to interfere with cell proliferation, their uptake and their effects on parameters of cellular state in human hepatoma cells (HepG2).

Cell growth experiments show that resveratrol triacetate presents a slightly better antiproliferative potential than resveratrol. The dimer  $\epsilon$ -viniferin, as well as its pentaacetate analogue, is less powerful than resveratrol, although a similar uptake kinetics in cells. Interestingly, among the tested polyphenols, vineatrol is the most potent solution, indicating a possible synergistic effect of both resveratrol and  $\epsilon$ -viniferin. We took advantage of the fluorescence properties of these compounds to evidence cellular uptake by using flow cytometry. In addition, by competition assay, we demonstrate that resveratrol triacetate enters in hepatic HepG2 cells by the same way as resveratrol. By autofluorescence *in situ* measurement we observed that resveratrol and related compounds induce deep changes in

cells activity. These changes occur mainly by increasing NADPH cell content and the number of green fluorescent cytoplasmic granular structures which may be related to an induction of detoxifying enzyme mechanisms.

**Keywords:** resveratrol,  $\epsilon$ -viniferin, acetate derivatives, cell uptake, cell proliferation, autofluorescence.

**Correspondence :** \*Norbert Latruffe, INSERM U 866, Université de Bourgogne, Équipe Biochimie Métabolique et Nutritionnelle, Faculté des Sciences Gabriel, 6 Bd Gabriel, F-21000 Dijon, France. Fax: 333.80.39.62.50. Email [latruffe@u-bourgogne.fr](mailto:latruffe@u-bourgogne.fr)

## 1 - Introduction

Resveratrol is a well known polyphenol present in various plant species, and particularly in grapevine [1]. It exhibits several beneficial effects for health i.e. protective properties against cardiovascular diseases [2, 3], cancer [4-6], viral attacks [7], but also towards neurodegenerative diseases [8] and ageing [9]. In addition, it increases lifespan [10] and physical activity [11, 12].

Resveratrol is a strong antioxidant and a free radical scavenger [13]. The structural basis of these properties has been related to the molecular packing in the solid state showing extensive hydrogen bound network [14]. Antioxidant activity is considered to be essential in the prevention of chemical-induced cancer by inhibiting initiation step of carcinogenesis process [15].

On the other hand, natural resveratrol oligomers have been found in various plants including species used in Chinese medicine. Some of these oligomers have been tested for their antioxidant properties [16, 17]. However, among them  $\epsilon$ -viniferin, a resveratrol dimer present in grapevine and in wine has been the subject of few studies. This resveratrol dimer was shown to inhibit cytochrome P450 enzymes [18] and to have antiproliferative and apoptotic effects on leukemic cells [19, 20]. *In vivo*, the protective effect of  $\epsilon$ -viniferin against hepatic injury has been shown in mice [21].

Interestingly, some synthetic acetate derivatives of resveratrol and of its oligomers have been prepared. The hypothesis of a possible lipophilicity increase may modify the transport and the uptake of the parent molecule, as widely described for phenolic and other compounds [22].

While the literature on the effects of resveratrol on cancer cells is abundant, numerous data report a poor bioavailability of this molecule [23]. At the opposite, data on the antiproliferative effect of resveratrol derivatives are scarce, the aim of this study is to compare the potencies of *trans*-resveratrol and *trans*- $\epsilon$ -viniferin and their respective acetate derivatives (figure 1) as well as a mixture of *trans*-resveratrol, *trans*- $\epsilon$ -viniferin so-called vineatrol extracted from grapevine shoots. This work presents their abilities to reduce the proliferation of human tumor hepatic cells and to penetrate such cells. The perspectives of the results in the cancer field are discussed. Moreover *in situ* cellular autofluorescence measurements allow to estimate disturbance of treated cells with resveratrol and resveratrol triacetate.

## 2 - Material and methods

### *Cell culture and treatments*

HepG2 cell line is a human hepatoma cell line which exhibits high metabolic capacities towards xenobiotics and polyphenols, and which is also a cancerous cell line. We previously reported resveratrol antiproliferative effects and metabolism in this cell line [24-26]. HepG2 cell line obtained from the American Tissue Culture Collection (ATCC, Rockville, MD) were maintained in DMEM (Sigma-Aldrich, France), complemented with 10% fetal calf serum. Cells were treated by diluting 30 mM ethanolic stock solutions of the different polyphenols in DMEM medium containing 0.1% ethanol (v/v). *Trans*-resveratrol (R) was obtained from Sigma-Aldrich (France); *trans*-resveratrol triacetate (3,5,4'-triacylresveratrol) (R3A),  $\epsilon$ -viniferin ( $\epsilon$ V),  $\epsilon$ -viniferin pentaacetate ( $\epsilon$ V5A) and vineatrol were a gift from Actichem (Montauban, France). Treatments by vineatrol, a mixture of polyphenols derived from grapevine shoots [19] containing 16 % *trans*-resveratrol and 20 %  $\epsilon$ -viniferin, were performed with respect to its content in *trans*-resveratrol : vineatrol effects were compared to 30  $\mu$ M *trans*-resveratrol effects by treating cells with doses of vineatrol containing 10  $\mu$ M (Vinea10) and 30  $\mu$ M resveratrol (Vinea 30).

### ***Proliferation assays***

HepG2 cells ( $1 \times 10^5$ ) were seeded into 24-well plates in 1 mL of culture medium. After 48h, the cells were treated in triplicate wells with polyphenols at the indicated concentrations (see legends of figures). Control cells were exposed to the same final concentration of ethanol (0.1%). Adherent cells and detached cells were collected and pooled after 24, 48 and 72h. The cell growth extent was determined by counting viable cells by trypan blue dye exclusion with a haemocytometer. IC<sub>50</sub> were determined by counting viable HepG2 cells after 48h treatments with polyphenols at 1, 5, 10, 30, 60 and 100  $\mu$ M in triplicate wells.

### ***Tritiated resveratrol uptake measurement***

[<sup>3</sup>H]-*trans* resveratrol (specific activity: 74 GBq/mmol) labeled in *ortho* and *para* of benzenic rings, was prepared for us by Amersham. Resveratrol uptake was examined by a 10 minutes cell incubation at two different temperatures (37°C and 4°C) with medium or HBSS containing tritiated resveratrol at various concentrations, with cold resveratrol or resveratrol triacetate in a 20-fold excess concentration. As previously reported [25], the labeled medium was removed at the end of the incubation period, and the cell wells were washed three times with a cold PBS buffer. Then, the cells were lysed by a lysis buffer (0.1% SDS, 0.1 M NaOH, 2% Na<sub>2</sub>CO<sub>3</sub>, 400  $\mu$ L per well). The cell homogenates were transferred into flasks and cell-associated radioactivity was counted in a liquid scintillation analyzer. The radioactivity of

collected cell media was also determined in aliquots.

### ***Spectrofluorimetry***

Excitation and emission spectra of *trans*-resveratrol, resveratrol triacetate,  $\epsilon$ -viniferin and  $\epsilon$ -viniferin pentaacetate, at 10  $\mu$ M in ethanol, were recorded using a Kontron SFM25 spectrofluorimeter, equipped with a 1 cm quartz cell. The absorbances of the solutions were always lesser than 0.05, allowing a correct measure in spectrofluorimetry.

### ***Flow cytometry analyses***

HepG2 cells ( $1 \times 10^5$ ) were seeded into 24-well plates in 1 mL of culture medium. After 48h, cells were treated in triplicate wells with 10, 30 and 60  $\mu$ M of resveratrol, resveratrol triacetate,  $\epsilon$ -viniferin and  $\epsilon$ -viniferin pentaacetate in culture medium. After 1, 2, 5, 10, 30 minutes, 3h, 6h, 24h and 48h of incubations, cells were quickly washed with cold PBS, scrapped, dissociated and immediately submitted to flow cytometry evaluation of emitted fluorescence. Polyphenols associated autofluorescence in HepG2 cells was measured with a Galaxy flow cytometer (Dako/Partec, Münster, Germany) equipped with a 350 nm UV lamp. Fluorescence emissions were measured through a 420 nm wavelength long-pass filter. Polyphenol-induced fluorescence emission was measured by using an argon-ion laser emitting a 488 nm wavelength at the power of 20 mW. In this condition, fluorescence emission was measured through a 520 nm band-pass filter.

### ***NADPH quantification***

NADPH quantification was performed using the NADPH quantification kit from BioVision Inc. NADPH extraction was performed according to manufacturer. Briefly, HepG2 cells ( $1 \times 10^5$ ) were seeded into 24-well plates in 1 mL of culture medium. After 48h, cells were treated in triplicate wells with 30 and 60  $\mu$ M of resveratrol, resveratrol triacetate,  $\epsilon$ -viniferin and  $\epsilon$ -viniferin pentaacetate in culture medium. After 3 and 6h, cells were lysed using 200  $\mu$ L of extraction buffer and heated for 30 minutes at 60°C. Then, 50  $\mu$ L of the extracts were transferred in duplicates in 96-wells microplates and submitted to the action of 100  $\mu$ L of the provided cycling enzyme in its buffer during 5 minutes. After 1 to 3 hours of development obtained by addition of 10  $\mu$ L developer, microplates were read for absorbance at 450 nm. Results were expressed as NADPH picograms per micrograms proteins by plotting a NADPH standards curve and also by quantifying proteins in extracts by the Bradford method.

### ***Fluorescence microscopy***

Cytospin slides were prepared with untreated and treated HepG2 cells. Slides were submitted to spectral analysis under a 488 nm excitation by means of confocal laser scanning microscopy (CLSM) as previously described [27-30]. Briefly, sequences of images were acquired on a Leica TCS SPL equipped with a UV/visible laser (Spectra Physics 2018, Spectra Physics, Mountain View, CA) and analyzed for emitted spectra from 515 to 695 nm. The sequences were processed using the FAMIS algorithm (Factor Analysis of Medical Image Sequences) resulting in precise *in situ* analysis of cell fluorescence [31].

### ***Statistical significance***

Unless indicated in the legends of figures, the reported values represent the means of triplicate from one representative experiment repeated three times  $\pm$  SD. Statistical significance was determined using Mann-Whitney test at  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ .

## **3- Results**

### ***Comparative antiproliferative effects of resveratrol and its derivatives***

To clarify the potential antiproliferative effects of resveratrol analogues on cancer cells, we tested their activity on the HepG2 human hepatoma cell line as compared to the effect of resveratrol. As previously described by our team [24], the figure 2A shows that the treatment of HepG2 cells with resveratrol leads to a dose- and time-dependant inhibition of cell proliferation. The antiproliferative effect of  $\epsilon$ -viniferin (dimer of resveratrol) is not significant at 30  $\mu$ M. However, at 60  $\mu$ M (figure 2A),  $\epsilon$ -viniferin not only completely inhibits cell proliferation but kills the cells. The toxic effect is twice more than resveratrol at the same concentration (data not shown). Interestingly, vineatrol disturbs cells proliferation in a more efficient manner than resveratrol does. Indeed, vineatrol 10 (10  $\mu$ M resveratrol, 6.2  $\mu$ M  $\epsilon$ -viniferin) also gave a significant antiproliferative effect by decreasing cell growth by 37% whereas 10  $\mu$ M resveratrol or 6.2  $\mu$ M  $\epsilon$ -viniferin separately have no effect on HepG2 cells proliferation (data not shown). Moreover, treatments with vineatrol 30 in an equivalent of 30  $\mu$ M resveratrol and 18.7  $\mu$ M  $\epsilon$ -viniferin, inhibited 86% of cell proliferation compared to 75% for 30  $\mu$ M resveratrol alone.

Figure 2B reports the effect of acetylation on resveratrol and  $\epsilon$ -viniferin molecules on cell proliferation inhibition. The effect of resveratrol triacetate was more relevant and significant. With the same toxicity than 30  $\mu$ M resveratrol, its triacetate analogue exhibits at the same

concentration a significantly stronger inhibition of cell proliferation. Indeed, a 47% cell proliferation inhibition was obtained with resveratrol triacetate after 48h compared to 26% for resveratrol. In contrast, acetylated  $\epsilon$ -viniferin ( $\epsilon$ -viniferin pentaacetate), does not show any significant effect.

Dose effect curves (after 48h treatments) of the different polyphenols (figure 2C) clearly highlight the potentials of resveratrol triacetate which exerts stronger antiproliferative effects on HepG2 cells than resveratrol with an  $IC_{50}$  of 31.3 against 39.7  $\mu$ M for resveratrol.  $\epsilon$ -viniferin shows a nearly 1.5-fold lower activity than resveratrol with an  $IC_{50}$  of 58.4  $\mu$ M, whereas its pentaacetate derivative is quite more active ( $IC_{50}$  of 49.5  $\mu$ M). Vineatrol is also the most potent tested treatment with an  $IC_{50}$  of 16.9.

### ***Competitive effects of resveratrol triacetate on tritiated resveratrol uptake***

Following experiments of cell proliferation inhibition abilities, here we wanted to know if resveratrol analogues were able to penetrate into the cells since we have previously shown that resveratrol uptake occurs both by passive diffusion and by a facilitated process [25]. Concerning resveratrol analogues, we could not perform a direct study of their uptake, due to the lack of radiolabeled reference compounds. Therefore, we studied the effect of unlabelled resveratrol triacetate on radiolabeled resveratrol uptake (figure 3). Results using unlabeled resveratrol confirmed that a 20-fold excess over the radiotracer exerted no inhibition on passive process measured at 4°C, whereas it produces a 40% inhibition of the tracer uptake at 37°C. Interestingly, we observed a 30 % inhibition of tritiated resveratrol uptake at 37 °C in presence of 20-fold excess of resveratrol triacetate. No inhibition was observed in the incubations performed at 4°C, a temperature where only passive diffusion is taken into account. This competitive effect of resveratrol triacetate on the temperature-dependent resveratrol-uptake was similar to that obtained by using an excess of unlabelled resveratrol. This means that the acetylated resveratrol derivative would be taken up through an active process, involving the same transporter than the parent molecule.

### ***Fluorescence properties of resveratrol and analogues - Application to flow cytometry studies of these polyphenols uptake***

The excitation and emission spectra of resveratrol,  $\epsilon$ -viniferin and their acetylated analogues are reported in figure 4. The maximal excitation wavelength and the maximal emission wavelength of each compound were determined in ethanol solution (10  $\mu$ M final concentration). Excitation spectra (figure 4A) of both polyphenols, resveratrol and  $\epsilon$ -viniferin

and their acetylated forms, show two major peaks; one from 315 to 350 nm, corresponding to aromatic nuclei; the second one broader from 400 to 450 nm, corresponding to conjugated double bonds. As expected, the  $\epsilon$ -viniferin molecules present high excitation capacity in UV absorption related to their number of phenol groups. The acetylation also leads to a shift of 15 to 25 nm in the UV short wavelengths. Emission spectra (figure 4B) after excitation at 330 nm show a large peak in the 350 to 450 nm range for both polyphenols. As expected, their fluorescence intensities are related to the number of phenols groups and their acetylations. These spectra indicate that the intrinsic fluorescence of these compounds can be directly visualized by flow cytometry analyses by using the appropriated excitation wavelength and emission filters, allowing studies of intracellular uptake of the tested molecules.

### ***Time course of resveratrol uptake and its analogues***

According to fluorescence properties of resveratrol and its analogues above described (figure 4), we followed their intracellular fluorescence in HepG2 cells. Evaluation of polyphenols uptake by flow cytometry was performed by exciting cells with a UV lamp and acquiring emitted fluorescence through a FL4-420 nm long pass filter. The diagrams (figure 5) show time and dose-dependent variations of polyphenols induced fluorescence after UV excitation. This phenomenon appears in two waves. Firstly, we observe a rapid fluorescence increase during the first 30 minutes which are due to the polyphenols uptake. The fluorescence increases occur in different ways depending on treatments concentrations: in all cases, 10  $\mu$ M treatments induce a 10 minutes peak whereas higher concentrations delay this maximum until 30 minutes. According to our previous study on resveratrol [25], metabolism of low concentrations of tested polyphenols is thus quickly achieved by HepG2 cells. Moreover, fluorescence variations are dependent of the tested polyphenols, treatment with  $\epsilon$ -viniferins lead to a 30% shift of cells fluorescence compared to resveratrol. We explain this phenomenon by the number of aromatic nuclei in the tested polyphenols which are responsible of their intrinsic fluorescence as shown in figure 4. The acetylations also increase the respective maxima of parent molecules.

Secondly, after 1h, polyphenols induce another increase of HepG2 cells fluorescence under UV excitation. This phenomenon does not seem to be due to polyphenols intrinsic fluorescence since their uptake and metabolism occurs before this time. Nevertheless, this cell autofluorescence is correlated to polyphenols dose treatments and may reflect changes in cellular metabolism. This induced autofluorescence reaches its maximum between 3h and 6h and stays quantifiable until 48h in resveratrol related compounds treated cells.



### ***Quantification of NADPH cell content induction by resveratrol and its analogues***

Flow cytometry analyses by UV excitation reveal that resveratrol related compounds induce an increase of cells autofluorescence. It is also known that reduced pyridine nucleotide NAD(P)H is the major intracellular fluorophore which presents a 338 nm excitation maximum and emits in the range of 440 to 480 nm [32-34]. We also decided to quantify the consequences of resveratrol related compounds treatments on cellular NADPH content. This determination was performed after 3h and 6h treatments since the maximum cell autofluorescence was measured at these times (figure 5). These quantifications reveal an average increase of HepG2 cells NADPH content of nearly 1.4 fold compared to control after 3h treatments by polyphenols. After 6h treatments, this increase reaches approximately 1.1 fold. The enhancement of cellular NADPH is time dependent, but it only tends to differ between polyphenols tested doses. These results correlates with UV flow cytometry analyses.

### ***Evaluation of cell responses induced by resveratrol and its analogues studied by intrinsic cell autofluorescence time course***

Following the cell antiproliferative effect of resveratrol and analogues and their penetration in the intracellular compartment, we studied the cell response by recording their changes in intrinsic autofluorescence and in side scattering correlated to granularity (figure 7). We commonly observed that several cell lines present an induced spontaneous green fluorescence increase after resveratrol treatment (unpublished results). So we report quantification of this phenomenon here. Indeed, treatments of HepG2 cells with resveratrol at various concentrations produce a time- and dose-dependent increase of spontaneous cell autofluorescence (figure 7A). The green emitted fluorescence ( $520\pm 10$  nm) after 488 nm excitation does not overlap resveratrol fluorescence and took place after 24h resveratrol treatment ( $60\ \mu\text{M}$ ). Interestingly autofluorescence is higher for resveratrol triacetate at a lower concentration ( $30\ \mu\text{M}$ ). After 48h of treatment, cells autofluorescence is significantly higher for all resveratrol treatments. However at this treatment length, the resveratrol triacetate ( $30\ \mu\text{M}$ )-induced cells autofluorescence strongly decreases to reach the same one than the equivalent resveratrol (figure 7A).  $\epsilon$ -viniferin and its pentaacetate derivative do not induce modifications in cells autofluorescence (not shown).

Flow cytometry data were confirmed using spectral analysis by confocal laser scanning microscopy. Cells treated for 48h with resveratrol ( $10$ ,  $30$  or  $60\ \mu\text{M}$ ) and its triacetate analogue ( $30\ \mu\text{M}$ ) were submitted to 488 nm excitation and the emitted fluorescence was

recorded from 515 to 695 nm. From this, FAMIS algorithm allowed to plot *in situ* emission spectra [31]. The spectra revealed (figure 7B) that resveratrol and resveratrol triacetate induce a 4 to 5-fold green fluorescence increase whereas no changes in cells were observed after  $\epsilon$ -viniferins treatments. In addition, microscopic observations showed that this induced autofluorescence is cytoplasmic and appears in a diffuse granular pattern.

Flow cytometry analysis (figure 7C) also revealed a strong increase in side scatter parameter after resveratrol or resveratrol triacetate treatment. This increase was time- and dose-dependent (results not shown) and corresponds to deep changes in cells morphology and granularity. In an unexpected manner, no effects were seen with  $\epsilon$ -viniferin and  $\epsilon$ -viniferin pentaacetate, suggesting different action mechanisms between resveratrol and its dimeric form on cell disturbance.

#### **4. Discussion**

We have previously investigated the mechanisms underlying the antiproliferative effects of resveratrol. Indeed, this polyphenol is able to inhibit HepG2 cells proliferation in a time- and dose-dependent manner by blocking their progression in their cell cycle at the G2/M transition [24] and by modulating NO production by increasing iNOS and eNOS expression and activity [35]. Cell cycle blockade in cancer cells occurring after resveratrol treatments is widely described and attributed to cyclins-cdks complexes induced disturbance [36]. Other works have evidenced that resveratrol acts by modulating many targets activities including COX-2, growth factor pathways (TNF, EGF...), NFkB, polyamine metabolism or VEGF (for review see [4]). Although mechanism are not fully elucidated, the aim of this study was to show that, in addition to resveratrol, other compounds produced by grape may exert an inhibitory effect on the proliferation of human liver tumor cells. We also emitted the hypothesis that  $\epsilon$ -viniferin, a dimer of resveratrol and acetylation of polyphenols can increase anticancer properties of these molecules.

Several resveratrol oligomers were identified in different plants in the last years and the cellular properties of some of them have been investigated [16, 19, 37-53]. Related to the effect of resveratrol oligomers on cell cycle inhibition and apoptosis,  $\epsilon$ -viniferin induces apoptosis and cell cycle modifications in leukemia cells [19, 42], the resveratrol trimer alpha-viniferin inhibits keratinocyte proliferation [44]; gnetin H and suffriticosol induce apoptosis in leukemia HL 60 cells [42]. The tetramers vaticanol inhibit prosurvival pathway [49] and hopeaphenol exhibits cytotoxic properties [50]. Antiproliferative potential of the resveratrol

dimer  $\epsilon$ -viniferin against cancer cells was not well studied and we also decided to test this polyphenol on a solid tumor cell line growth. This molecule exhibits an antiproliferative potential on HepG2 cells mainly due to a cytotoxic effect compared to resveratrol.

Acetylated polyphenols are of potential interest due to their higher hydrophobicity than the parent molecules, being able to cross the membrane more easily [22, 50]. In accordance to this, we show that the acetylation of resveratrol led to a greater inhibitory effect on HepG2 cell growth. Conversely, on prostate tumor cell line, it was reported that the cell-growth inhibition activities of triacetate but also tributanoate and trioctanoate derivatives of resveratrol were similar to that of resveratrol [54]. Thus, these derivatives seem to present a tumor-type selective activity, as resveratrol. In a same way,  $\epsilon$ -viniferin acetylation modestly improves the antiproliferative effect of the parent molecule.

Interestingly, the effect of vineatrol is greater than the expected additive effect of resveratrol and  $\epsilon$ -viniferin, at equivalent concentrations. It was reported that a preparation of 10% vineatrol exhibited a greater antiproliferative effect than resveratrol or  $\epsilon$ -viniferin on lymphocytic leukemia cells [19]. It cannot be excluded that the great effect of vineatrol would be related to the presence of other resveratrol oligomers in this grapevine shoot extracts, but some of these compounds have been only found in traces in vineatrol (J-C Izard, personal communication).

To go further from the results obtained with these new polyphenols, we studied their cell penetrability by using radiolabelling competitive experiments and polyphenol fluorescence assay. This work reports that resveratrol derivatives,  $\epsilon$ -viniferin and resveratrol triacetate penetrate in the cell as it is for resveratrol [25]. Thus, the uptake of resveratrol triacetate occurs according to two processes, a passive one and a carrier-mediated one. We have not yet identified the protein(s) responsible for the facilitated uptake of resveratrol and resveratrol triacetate. Han et al. [55] showed in the rat brain the presence of specific plasma membrane binding sites for polyphenols including resveratrol. Such binding sites could be present in plasma membrane of hepatic cells too. Furthermore, we have shown that the efflux of resveratrol across HepG2 cells occurs in a sulfoconjugated form and presumably through a plasma membrane MRP [26]. In contrast, resveratrol metabolism occurring in rodents leads to glucuronoconjugated forms (for a review, see [56]). This work also show that tested polyphenols metabolism is dose-dependent, high concentrations of them leading to delay their efflux, mainly by saturating their metabolism as reported by Maier-Salamon et al. [57].

Despite  $\epsilon$ -viniferin has a lower antiproliferative effect toward HepG2 cells, its uptake kinetic is similar to resveratrol. The higher hydrophobicity procured by acetylation of the phenol

groups enhances in a significant manner, although not dramatically, the cell uptake rate of resveratrol and  $\epsilon$ -viniferin as expected: they would easily cross the membrane bilayers. The plasmatic proteins, which are largely hydrophilic, should bind the acetylated derivatives with a lower affinity than the parent molecules. We have indeed shown that resveratrol may be largely bound to plasmatic proteins, in particular albumin [58]. Acetylated forms are hydrolyzed by intracellular esterases and further conjugated as the parent molecules. We have shown such pathway for resveratrol triacetate on HepG2 cells (data not shown).

In the last part of this study, we focused on phenotypical cells changes occurring after resveratrol and its derivatives treatments. We previously observed increasing cell fluorescence background using fluorescence technique in different cell types after polyphenols treatments. This induced autofluorescence occurs in a time- and dose-dependent manner. It is not due to polyphenols themselves or to their metabolites because of their low 488 nm-excitation properties, and their rapid excretion before 1h. We report here that HepG2 cells induced green autofluorescence occurs concomitantly to polyphenols antiproliferative potential: this phenomenon only occurs after resveratrol or after acetylated analogue treatments. After 1h of polyphenols treatments, cells also emit in blue wavelength after UV-excitation. Autofluorescence of cells is documented and also used to determine metabolic state of cells, mainly hepatic cells [33, 59]. The high fluorescent is reported to be due to the fluorescence properties of reduced pyridine nucleotide NAD(P)H (emitting in the range of 440 to 480 nm after UV-excitation) and flavins FAD/FMN (emitting in the range of 520 nm after 488 nm-excitation) as major intracellular fluorophores [32, 34]. By the way, resveratrol and viniferin (resveratrol dimer) don't seem to have similar targets since only resveratrol and resveratrol triacetate induce green cells autofluorescence. We also show here that cellular NADPH level increases after polyphenols treatments in HepG2 cells in a time-dependent manner. Related to this, it is well known that many endoplasmic reticulum membrane bound enzymes are NADP(H) or FAD dependent enzymes. In addition, we recently reported that resveratrol induces detoxifying processes by activation of gene encoding UDP-glucuronosyltransferases and sulfotransferases, two groups of endoplasmic reticulum membrane bound enzymes [26]. Resveratrol also stimulates cellular metabolism [11, 12]. We hypothesize that this autofluorescence induction are related to such mechanisms. Microscopic analyses revealed that this resveratrol induced spontaneous fluorescence which takes place in the cytoplasmic compartment showing green granular-like accumulations. This observation can be linked to above described phenomenons which could occur in the endoplasmic reticulum and in the mitochondria. We report that these resveratrol induced subcellular changes can be monitored

by phenotypical analyses of cell structure using flow cytometric side scatter parameter, which increase during resveratrol treatments. All these induced subcellular changes must be kept in mind by experimenters, mainly when using fluorescence techniques to study drugs action mechanisms.

In conclusion, this *in vitro* study shows that resveratrol analogues and polyphenol mixtures show more efficient antiproliferative effects than resveratrol. It also demonstrates that polyphenols accumulation and subsequent cellular effects vary from one compound to another. Since reports indicate that the resveratrol tissue concentration is probably higher or in the same range of order as concentrations *in vitro* tested [60, 61], the bioavailability and cellular effects of our tested molecules have to be further achieved by *in vivo* approach.

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