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In Vitro Safety/Protection Assessment of Resveratrol and Pterostilbene in a Human Hepatoma Cell Line (HepG2)

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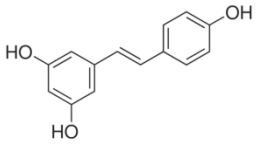
The aim of this work was to evaluate *in vitro* the genotoxic and/or antigenotoxic effects of resveratrol (RESV) and pterostilbene (PTER) on HepG2 cells. Moreover, additional tests were performed to evaluate early and late apoptosis events induced by the tested stilbenes. RESV and PTER did not show any genotoxic activity. As regards antigenotoxicity testing, RESV and PTER showed a typical, U-shaped hormetic dose-response relationship characterized by a biphasic trend with small quantities having opposite effects to large ones. HepG2 cells treated with PTER exhibited a marked increase in early apoptosis (40.1 %) at 250 μM; whereas, the highest concentration tested for both RESV and PTER significantly increased the proportion of HepG2 cells undergoing late apoptosis (32.5 and 51.2 %, respectively). The observed pro-apoptotic activity could, at least in part, explain the hormetic response observed when the compounds were tested for antigenotoxicity (*i.e.*, in the presence of induced DNA damage).

Keywords: Resveratrol, Pterostilbene, Genotoxicity, Antigenotoxicity, Comet assay, Apoptosis.

The stilbene scaffold is known to have several biological effects and nowadays, among them, *t*-resveratrol (RESV) (Figure 1/*A*) and *t*-pterostilbene (PTER) (Figure 1/*B*) are especially studied for their potentials for human health. RESV and PTER are naturally occurring phytoalexins found in a wide range of plant families, including *Vitis* and *Vaccinium* and, therefore, in derived foods such as grapes, wine and blueberries. Moreover, for their interesting biological properties, they are present in commercial dietary supplements. In particular, they have been reported to have antioxidant, anti-inflammatory, antibiotic and neuroprotective effects and their ability to inhibit the proliferation of a variety of human cancer cell lines has also been demonstrated [1-4]. Indeed, the interaction of reactive species of a carcinogen with DNA can result in genotoxic damage and so, protection from genotoxicity is an event that could be considered as a mechanism to prevent carcinogenesis. In particular, RESV has been shown by Comet assay to prevent oxidative DNA damage induced by different stimuli and, at the same time, it has been reported that at high doses it induces *in vitro* apoptosis in cancer cells, as well as PTER [5, 6]. The apoptosis phenomenon could be associated with an oxidative DNA strand breakage and, effectively, most plant polyphenols show both antioxidant and prooxidant properties. On the other hand, it has been proposed that the prooxidant action of these compounds may be an important mechanism of their anticancer properties [7-15].

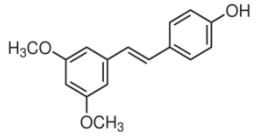
Even if RESV and PTER have similar biological activity, they show different bioavailability due to their different chemical structures (Figure 1, *A* and *B*): RESV is a 3,5,4'- trihydroxystilbene, whereas its methoxylated analogue, PTER, is a 3,5-dimethoxy-4'-hydroxy stilbene [16, 17]. Thus, since RESV has three hydroxy groups and its analog has one hydroxy and two methoxy groups, it has a lower metabolic stability than its analog. Moreover, the presence of methoxy groups causes PTER to be more lipophilic and consequently more permeable across biological membranes increasing thus its pharmacokinetic profile. Indeed, when administered orally, PTER shows 95% bioavailability, but RESV only 20%. Besides, PTER's half-life is seven times longer than RESV, 105 and 14 min, respectively [18-21].

(A)



Synonym:
3,5,4′-trihydroxy-*trans*-stilbene
IUPAC name:
5-[(*E*)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol
CAS registry No.: 501-36-0
Molecular formula: $C_{14}H_{12}O_3$
Molecular Weight: 228.24

(B)



Synonym:
3,5-dimethoxy-4′-hydroxy-*trans*-stilbene
IUPAC name:
4-[(*E*)-2-(3,5-dimethoxyphenyl)ethenyl]phenol
CAS registry No.: 537-42-8
Molecular formula: $C_{16}H_{16}O_3$
Molecular Weight: 256.30

Figure 1: *t*-Resveratrol (*A*) and *t*-pterostilbene (*B*) chemical data.

The aim of this work was to evaluate *in vitro* the genotoxic and/or antigenotoxic effects of RESV and PTER on HepG2 cells. In order to understand the mechanism of protective action (*i.e.*, antigenotoxic) of stilbenes, cell cultures were treated with the tested compounds during and before treatment with the model mutagen 4-nitroquinoline-*N*-oxide (4NQO) to follow co-exposure or pre-exposure protocols. The single-cell microgel-electrophoresis (Comet) assay is a simple and fast technique allowing the detection of DNA strand breakage [22]. For this test, a suspension of isolated cells is embedded into an agarose microgel onto a microscope slide and subsequently lysed by detergents at high salt concentration. The liberated DNA is then exposed to alkali for unwinding from the strand breakage sites and electrophoresed under alkaline conditions. In the presence of DNA strand breaks, electrophoresis at high pH and staining with ethidium bromide result in structures resembling comets with the tail length or tail fluorescence content reflecting the frequency of DNA strand breaks and hence DNA damage. The standard alkaline procedure (lysis at pH 10, unwinding and electrophoresis at pH >13) allows the detection of both single- and double-strand DNA breaks, as well as apurinic/apirimidinic sites (*i.e.*, alkali labile lesions) that are expressed as frank strand breaks in the DNA under the alkaline conditions of the assay; moreover, incomplete excision repair sites are also detected by the alkaline Comet assay.

Moreover, additional tests were performed to evaluate early and late apoptosis events induced in HepG2 cells by RESV and PTER. Loss of the mitochondrial membrane potential ($\Delta\psi m$) is known to precede apoptosis and chemical-hypoxia-induced necrosis. The lipophilic cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) displays potential-dependent accumulation in the mitochondria and provides a fluorescent-based method for distinguishing between healthy and apoptotic cells. In healthy cells, in fact, the negative charge established by the intact mitochondrial membrane potential facilitates the accumulation of JC-1 in the mitochondrial matrix. At high concentrations, JC-1 forms aggregates and become red fluorescent. As an early event, in apoptotic cells the mitochondrial potential collapses and JC-1 localizes to the cytosol in its monomeric green fluorescent form [23]. During apoptosis calcium- and magnesium-dependent nucleases are activated to degrade DNA [24, 25]. The consequence of nicks and double-stand breaks formed along the DNA molecule is DNA fragmentation. This late event of apoptosis was detected by DNA content analysis to discriminate cells having less than 2 C DNA (DNA content of a diploid somatic nucleus), so-called sub-G_1 cells. The sub-G_1 method relies on the fact that after DNA fragmentation, small DNA molecules are able to diffuse out of the cells following washing. Thus, after staining with a quantitative DNA-binding dye, such as 6,4'-diamidino-2-phenylindole (DAPI), cells that have lost DNA will take up less stain and will appear left of the G_1 peak in a DNA content histogram.

To determine the cytotoxic effects of RESV and PTER, HepG2 cells were treated with five increasing doses of compounds for either 4 or 24 hours; cell viability was then determined by double staining of cells with acridine orange (AO) and DAPI. As shown in Table 1, after 4 h treatment of cells, only PTER at the highest tested dose (*i.e.*, 250 μM) significantly reduced the viability of HepG2 cells; after 24 h exposure, viability of HepG2 cells treated with the highest concentrations of PTER (*i.e.*, 100 and 250 μM) was significantly lower than that for control (untreated) cells.

In genotoxicity/antigenotoxicity tests, HepG2 cultures treated with serial dilutions of either RESV or PTER have never shown genotoxic effects, with mean tail intensity values similar to that for the negative control; the model mutagen 4NQO also served as a positive control and demonstrated the sensitivity of the Comet assay giving a clear positive response at the used concentration (data not shown).

Table 1: Viability (%), in HepG2 cells exposed *in vitro* for 4 or 24 h to *t*-resveratrol (RESV) or *t*-pterostilbene (PTER). Results expressed as the mean ± standard error (SEM) of independent experiments.

Concentration (μM)	Viability %			
	4 h		24 h	
	RESV	PTER	RESV	PTER
0	89.5 ± 4.5		93.5 ± 0.5	
10	90.7 ± 2.4	90.7 ± 3.3	85.0 ± 8.6	86.0 ± 4.7
25	89.7 ± 1.7	84.3 ± 4.5	89.7 ± 3.5	84.3 ± 7.4
50	90.0 ± 1.5	89.3 ± 2.9	85.7 ± 4.4	80.7 ± 8.7
100	87.3 ± 3.3	88.0 ± 2.1	84.3 ± 6.9	62.3 ± 15.8*
250	90.7 ± 2.7	31.0 ± 1.0*	71.7 ± 13.1	40.0 ± 19.0*

* $p < 0.05$ *vs*. negative control; one-way ANOVA.

Figure 2 summarizes the extent of primary DNA damage in HepG2 cell cultures concomitantly exposed to RESV (Figure 2/*A*) or PTER (Figure 2/*B*) and to 4NQO (antigenotoxicity testing; co-exposure).

Figure 2/*A* illustrates the antigenotoxic effect of RESV toward the extent of 4NQO-induced DNA damage (challenge cultures). HepG2 cells showed a U-shaped concentration-response curve of residual DNA, with RESV showing a slight antigenotoxic effect at concentrations of 10, 25 and 50 μM. Observed genotoxic inhibition rate (GIR%) values were 12.3 (± 16.5), 26.4 (± 21.8) and 33.6 (± 11.7) for the concentrations of 10, 25 and 50 μM, respectively, the latter concentration being the most effective. On the contrary, no antigenotoxic effects were observed for concentrations of 100 and 250 μM, the extent of DNA damage being higher than 4NQO (positive control), with negative GIR% values that clearly indicate an increased genotoxic activity of 4NQO in the presence of 100 and 250 μM RESV.

Figure 2/*B* summarizes the antigenotoxic effect of PTER toward the extent of 4NQO-induced DNA damage (challenge cultures); the highest concentration tested (*i.e.*, 250 μM) caused a massive loss of cells, probably because of cytotoxic effects. As regards antigenotoxic effects, co-exposure of HepG2 cells showed a U-shaped concentration-response curve of residual DNA damage with the highest effect at 50 μM concentration; the observed GIR% values were 34.7 (± 5.0), 48.7 (± 15.4), 55.09 (± 4.8) and 19.4 (± 25.9) for the concentrations of 10, 25, 50 and 100 μM, respectively.

Figure 3 shows the extent of primary DNA damage in HepG2 cell cultures pre-exposed for 4 h to either RESV or PTER and subsequently treated for 4 h with 4NQO (antigenotoxicity testing; pre-exposure).

Figure 3/*A* summarizes the antigenotoxic effects obtained by pre-treatment of cells with RESV toward the extent of 4NQO-induced DNA damage. Also, in this case, RESV showed a slight antigenotoxic effect at concentrations of 10, 25 and 50 μM, with GIR% = 24.5 (± 7.0), 30.8 (± 10.3) and 36.7 (± 13.5), respectively, the latter concentration being the most effective. For concentrations of 100 and 250 μM no antigenotoxic effects were observed, with a U-shaped concentration-response curve of residual DNA damage. Moreover, the negative GIR% value observed for the highest concentration tested also in this case indicates an increased genotoxic activity of 4NQO in the presence of 250 μM RESV.

Antigenotoxic effects of PTER in the pre-exposure protocol are summarized in Figure 3/*B*; the highest concentration tested (*i.e.*, 250 μM PTER) caused again a massive loss of cells, probably because

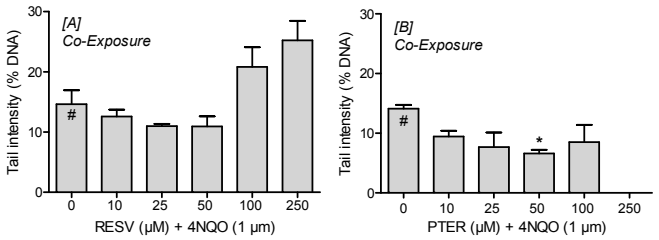


Figure 2: Antigenotoxic effects of RESV [*A*] or PTER [*B*] on 4NQO-induced DNA damage in HepG2 cells: co-exposure protocol. Each result is expressed as the mean ± standard error (SEM) of three independent experiments.
positive control (*i.e.*, 1 μM 4NQO, alone); * $p < 0.05$ *vs*. 4NQO, one-way ANOVA.

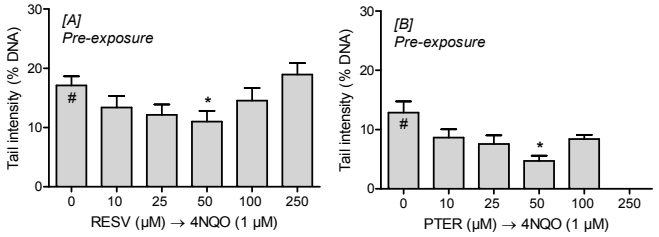


Figure 3: Antigenotoxic effects of RESV [*A*] or PTER [*B*] on 4NQO-induced DNA damage in HepG2 cells: pre-exposure protocol. Each result is expressed as the mean ± standard error (SEM) of three independent experiments.
positive control (*i.e.*, 1 μM 4NQO, alone); * $p < 0.05$ *vs*. 4NQO, one-way ANOVA.

Table 2: Percentage of apoptotic HepG2 cells after 4 or 24 h treatment with RESV or PTER: early (mitochondrial membrane potential; Δψm) and late (DNA fragmentation) apoptosis. Each value represents the mean ± SEM of three independent experiments.

Concentration (μM)	Depolarized Δψm (4 h treatment)		Fragmented DNA (24 h treatment)	
	% of depolarized cells		% of sub-G_1 cells	
	RESV	PTER	RESV	PTER
0	9.8 ± 0.7[a]		8.1 ± 1.1[a]	
10	12.3 ± 1.2	21.6 ± 4.9	6.1 ± 0.6	5.6 ± 1.1
25	16.6 ± 3.0	20.3 ± 1.6	10.3 ± 0.0	8.3 ± 0.5
50	20.6 ± 4.7	23.6 ± 7.1	9.6 ± 0.7	11.0 ± 0.8
100	23.9 ± 1.8	17.6 ± 1.9	14.9 ± 1.3	17.9 ± 0.3
250	12.6 ± 3.3	40.1 ± 11.1*	32.5 ± 1.2*	59.2 ± 1.8*
Positive control	25.1 ± 2.5[b]*		21.3 ± 1.9[c]*	

[a]Negative control: MEM, complete medium. [b]Positive control: 0.5 μM valinomycin.
[c]Positive control: 1 μM staurosporine.
* $p < 0.05$ *vs*. negative control; one-way ANOVA.

of cytotoxic effects. Pre-exposure of HepG2 cells with PTER resulted in a U-shaped concentration-response curve of residual DNA damage with the highest effect at a concentration of 50 μM; the observed GIR% values were 36.5 (± 5.3), 41.9 (± 7.9), 57.0 (± 12.8) and 37.2 (± 15.9) for the concentrations of 10, 25, 50 and 100 μM, respectively, with 50 μM PTER being the most effective antigenotoxic concentration.

As shown in Table 2, we found that, compared with the control group, PTER treatment produced a significant collapse of the mitochondrial membrane potential in cells exposed to the highest dose (*i.e.*, 250 μM), indicating an altered mitochondrial function after treatment with this compound. The results of a representative experiment illustrating the shift in fluorescence of JC-1 in HepG2 cells treated with either RESV or PTER relative to negative and positive control are shown in Figure 4.

The late event of apoptosis was detected by DNA content analysis. The results (Table 2) indicated that RESV- and PTER-treated HepG2 cells revealed an increase in the sub-G_1 apoptotic fraction at the highest concentrations tested, as compared with negative controls.

As regards antigenotoxicity testing, RESV and PTER showed a typical, U-shaped hormetic response, with hormesis that can be

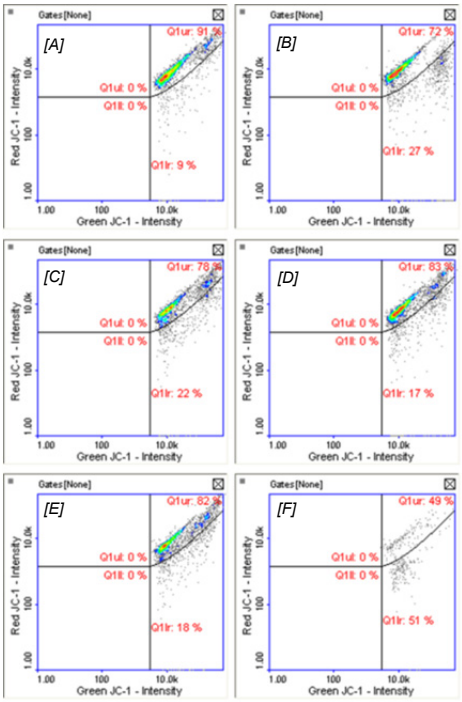


Figure 4: Determination of apoptosis (early events) by evaluation of mitochondrial membrane potential (Δψm). Green and red fluorescence of the viable cells displayed in a scatterplot: *[A]* negative control, *[B]* positive control (0.5 μM valinomycin), *[C]* 100 μM RESV, *[D]* 250 μM RESV, *[E]* 100 μM PTER, *[F]* 250 μM PTER.

defined as an adaptive, non-monotonic, biphasic dose-response relationship, characterized by small quantities having opposite effects than large quantities [26]. Biphasic dose-dependent effects have been largely reported in the literature, in particular for RESV that is known to induce hormetic responses in a wide range of biological models, affecting numerous endpoints of biomedical and therapeutic significance [27, 28].

In the present *in vitro* approach, toxic effects have been actually observed for both RESV and PTER in the experimental sets aimed at evaluating genotoxic/antigenotoxic properties of these *t*-stilbenes. In the co-exposure protocol (antigenotoxicity testing), for RESV concentrations of 100 and 250 μM the extent of DNA damage was higher than for 4NQO alone. PTER has always showed a marked cytotoxic effect at the highest concentration tested (*i.e.*, 250 μM).

As regards antigenotoxicity testing of the stilbene compounds, the outcomes demonstrated, in particular for RESV, that hormesis functions as a double-edged sword (antigenotoxicity/genotoxicity), with low RESV concentrations being potentially beneficial (antigenotoxic) and high concentrations having opposite, potentially harmful (pro-genotoxic) effects. Thus, the biological (e.g. biomedical, clinical) implications of the observed hormetic dose-responses need to be better clarified, as dietary supplementation success or failure is likely to be highly dependent on getting (individually) the right intake. In conclusion, on the basis of the reported results, in line with previously published data, it should be of pivotal importance in future or ongoing human-use trials of RESV (or other stilbenes) to take hormetic dose-responses into account to tailor doses to the individuals.

Moreover, HepG2 cells treated with PTER exhibited a marked increase in early apoptosis (40.1 %) at 250 μM; whereas, the highest concentration tested for both RESV and PTER significantly increased the proportion of HepG2 cells undergoing late apoptosis

(32.5 and 51.2 %, respectively). Apoptosis, or programmed cell death, is a normal physiologic process that occurs during embryonic development and in the ongoing process of tissue homeostasis in the adult animals. Any dysregulation of apoptosis can result in abnormality, disease and death [29]. The hallmarks of apoptosis include chromatin condensation, DNA fragmentation to nucleosome-sized pieces, membrane blebbing, cell shrinkage and compartmentalization of dead cells into membrane-enclosed vesicles or apoptotic bodies [30]. Cancer is a result of uncontrolled cell proliferation as well as the dysregulation of apoptosis [31] and it has been suggested that cancer chemotherapeutic as well as chemopreventive agents exert part of their pharmacological effects by triggering apoptotic cell death. The induction of apoptosis or arrest of cell cycle progression in tumor cells has become an indicator of tumor treatment response [32]. As a further conclusion, our results demonstrate that RESV and, particularly PTER are apoptotic in the HepG2 cell line when administered at high concentration. These data might suggest for these stilbenes a possible antitumor potential which can be attributed to their apoptotic actions against malignant HepG2 cells. A pro-apoptotic activity could, at least in part, explain the hormetic response observed when the compounds were tested for antigenotoxicity (*i.e.*, in the presence of induced DNA damage). However, prospective cancer-suppressive effects of the tested stilbenes should be further evaluated in *in vivo* experiments.

Experimental

Test compounds, chemicals and media: *t*-Resveratrol was purchased from Sigma-Aldrich Italia Srl (Milan, Italy), while *t*-pterostilbene was synthesized at the Department of Pharmaceutical Sciences (Unit of Organic Chemistry), University of Perugia (Perugia, Italy) according to a published procedure [33]. All reagents used were of analytical grade. Dimethyl sulfoxide (DMSO), ethanol (EtOH), ethylenediaminetetraacetic acid disodium (Na_2EDTA) and tetrasodium (Na_4EDTA) salt, hydrochloric acid (HCl), sodium chloride (NaCl), and sodium hydroxide (NaOH) were purchased from Carlo Erba Reagenti Srl (Milan, Italy). Ethidium bromide (EtBr), low- and normal-melting-point agarose (LMPA and NMPA, respectively), 4-nitroquinoline-*N*-oxide (4NQO), staurosporine, tris(hydroxymethyl)-aminomethane (Tris base), Triton X100, and valinomycyn were obtained from Sigma-Aldrich Srl (Milan, Italy). Gibco® Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), L-glutamine, antibiotics (*i.e.*, penicillin and streptomycin), sodium pyruvate, Dulbecco's phosphate buffered saline pH 7.4 (PBS), and trypsin were purchased from Invitrogen Srl (Milan, Italy), and acridine orange (AO), 6,4'-diamidino-2-phenylindole (DAPI) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1) from ChemoMetec A/S (Allerød, Denmark). Conventional microscope slides and coverslips were supplied by Knittel-Glaser GmbH (Braunschweig, Germany). Distilled water was used throughout the experiments.

Cell line: Human Caucasian hepatocyte carcinoma HepG2 cells were obtained from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna 'Bruno Ubertini' (Brescia, Italy). HepG2 cells retain many characteristics of hepatocytes such as the activities of phase I and phase II enzymes and reflect the metabolism of xenobiotics in the human body better than other metabolically incompetent cells [34-36].

The cells were grown as monolayer cultures in 25 cm^2 tissue flasks (Orange Scientific, Braine-l'Alleud, Belgium), in MEM added with 10%, v/v, heat-inactivated FBS, 2 mM *L*-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 0.1 mg/mL streptomycin. HepG2 cells were subcultured by dispersal with 0.05% trypsin in 0.02% Na_4EDTA for a contact time of 5 min and replated at a 1:2 dilution, which maintained cells in the exponential growth phase. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2; under these conditions, doubling time for HepG2 cells was about 24 h. For this study we used cells after 101-108 passages; cell stocks were routinely frozen and stored in liquid nitrogen.

Treatments for cytotoxicity, genotoxicity/antigenotoxicity and apoptosis testing were carried out in HepG2 cells subcultured in 6-well tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium) inoculated with 5 mL of complete MEM containing 5×10^5 cells per well.

Cytotoxicity testing: To examine cytotoxic effects eventually induced by RESV and PTER, changes in viability of HepG2 cells after 4 or 24 h exposure were analyzed by double staining of cells with AO and DAPI. A NucleoCounter® NC-3000™ (ChemoMetec A/S, Allerød, Denmark) automated system using fluorescence microscopy and image analysis was used to determine cell number and viability [37]. Double staining with 2 fluorescent dyes allows a cell stain (AO) for cell detection and a nucleic acid stain (DAPI) for detecting non-viable cells; DAPI cannot penetrate the cell membrane, hence it only stains cells with a permeable cell membrane (*i.e.*, non-viable cells). Briefly, Via1-Cassette™ (ChemoMetec A/S, Allerød, Denmark) were loaded with approximately 60 µL of a cell suspension. The inside of Via1-Cassettes is coated with the two fluorescent dyes, AO and DAPI, which were dissolved and stained, respectively, by the entire cell population (represented by green cells) and the non-viable cells (represented by blue cells). After placement of Via1-Cassettes in the NucleoCounter NC-3000, the stained cell samples were automatically measured, the fluorescent images recorded, and a report showing the results for cell counts and their viability generated.

Genotoxicity/antigenotoxicity testing - co-exposure treatment: After seeding for 48 h, in each well the culture medium was replaced by fresh complete MEM and the cells were then incubated for a further 4 h according to the following scheme:
(*i*) control cultures: fresh medium containing serial dilutions (10, 25, 50, 100 and 250 µM) of test compounds (*i.e.*, RESV or PTER);
(*ii*) challenge cultures: fresh medium containing serial dilutions (10, 25, 50, 100 and 250 µM) of test compounds plus 1 µM 4NQO.
Negative (*i.e.*, 1% DMSO) and positive (*i.e.*, 1 µM 4NQO) controls were included in each experimental set. Each experimental set was repeated at least 3 times. At the end of treatments, the cells were washed twice with 3 mL ice-cold PBS, pH 7.4, and detached with 300 µL of 0.05% trypsin in 0.02% Na_2EDTA. After 3 min, trypsinization was stopped by adding 700 µL complete culture medium. Cells were then collected by centrifugation (70×*g*, 8 min, +4°C).

Genotoxicity/antigenotoxicity testing - pre-exposure treatment: For 44 h the cells were maintained in complete MEM, then for both control and challenge cultures the culture medium was replaced by fresh complete growth medium containing different concentrations (10, 25, 50, 100 and 250 µM) of test compounds, and the cells were incubated for a further 4 h. After 4 h exposure, culture media containing RESV or PTER were removed and the cells incubated for a further 4 h according to the following scheme:
(*i*) control cultures: fresh medium;
(*ii*) challenge cultures: fresh medium added with 1 µM 4NQO.

Appropriate negative (*i.e.*, 1% DMSO) and positive (*i.e.*, 1 μM 4NQO) controls were included in each experimental set. Each experimental set was repeated at least 3 times. At the end of treatments, the cells were washed and harvested as above.

Alkaline single-cell microgel-electrophoresis (Comet) assay: Immediately after the exposure, HepG2 cells were processed in the Comet assay under alkaline conditions (lysis at pH 10, unwinding and electrophoresis at pH >13). The Comet assay was carried out basically following the original procedure [22, 38], with minor modifications [39]. Briefly, cell pellets were gently resuspended in 0.7% LMPA (in Ca^{2+}/Mg^{2+}-free PBS, w/v) maintained at 37°C. Then, 65 μL of cell suspension in agarose was rapidly layered onto pre-coated (1% NMPA, in Ca^{2+}/Mg^{2+}-free PBS, w/v) conventional 26×76 mm microscope slides and covered with a coverslip. After 10 min of agarose solidification at +4°C, the coverslips were removed and the microgels with the embedded cells covered with a top layer (75 μL) of 0.7% LMPA. After 10 min more of agarose solidification at +4°C the coverslips were further removed and the slides immersed in cold, freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl and NaOH to pH 10; 1% Triton X100 added just before use) overnight at +4ºC to obtain the lysis of cellular and nuclear membranes of the cells embedded in agarose microgels.

The slides were then drained and placed in a horizontal electrophoresis box (HU20, Scie-Plas, Cambridge, UK) filled with a freshly prepared electrophoresis solution (10 mM Na_4EDTA, 300 mM NaOH; pH > 13). After 20 min of preelectrophoresis to allow DNA unwinding and expression of alkali-labile damage, electrophoresis runs were performed in an ice bath for 20 min by applying an electric field of 25 V (1 V/cm) and adjusting the current to 300 mA (Power Supply PS250, Hybaid, Chesterfield, MO, USA). The microgels were then neutralized with 0.4 M Tris-HCl buffer (pH 7.5). For preservation, the slides were dehydrated in 70% ethanol (10 min), allowed to air-dry and stored in slide boxes at room temperature until ready to stain and analyze. All the steps of the Comet assay were conducted in yellow light to prevent the occurrence of additional DNA damage.

Computerized evaluation of DNA damage: Immediately before scoring, the air-dried slides were stained with 65 μL of 20 μg/mL EtBr and covered with a coverslip. The comets in each microgel were analyzed (blind), at 500× magnification, with an epifluorescent microscope (BX41, Olympus Co., Tokyo, Japan) under a 100 W high-pressure mercury lamp (HSH-1030-L, Ushio Inc., Tokyo, Japan), using appropriate optical filters (excitation filter 510-550 nm and emission filter 590 nm). The microscope, equipped with a high sensitivity black and white CCD camera (PE2020, Pulnix Europe Ltd., Basingstoke, UK), was connected to a computerized analysis system ("Comet Assay III", Perceptive Instruments, Suffolk, UK) that acquires images, computes the integrated intensity profile for each cell, estimates the comet cell components, head and tail, and evaluates a range of derived parameters. These include tail intensity (*i.e.*, percent of fluorescence migrated in the comet tail), which is considered to be the most useful parameter because of its linear relationship with strand-break frequency, and because it is relatively unaffected by threshold settings in the computerized analysis system [40]. A total of 100 randomly selected comets (50 cells/replicate slides) were evaluated for each experimental point. For each independent test, the median tail intensity of 50 cells/slide was assessed and the average of 2 replicated slides was calculated as a summary statistic [41].

Evaluation of mitochondrial membrane potential (Δψm) - early apoptosis assay: Mitochondrial membrane potential (Δψm) was estimated by using a NucleoCounter NC-3000 automated system after staining of cells with JC-1 and DAPI fluorescent dyes [42]. Mitochondrial depolarization was revealed as a decrease in the red/green fluorescence intensity ratio. In the test, necrotic cells were detected as blue fluorescent (DAPI) ones. The green and red fluorescence of the viable cells was displayed in a scatterplot. Gates in the scatter-plot were used to demarcate depolarized/apoptotic cells. Results are expressed as percentage of cells excluded by the gate. Valinomycin (0.5 μM) for 4 h was used as a positive control to depolarize the Δψm.

Evaluation of chromosomal DNA fragmentation - late apoptosis assay: Fragmentation of chromosomal DNA during late apoptosis was revealed by discrete sub-G_1 peaks on DNA content histograms [43]. A NucleoCounter NC-3000 automated system using fluorescence microscopy and image analysis was used to determine DNA fragmentation according to [42]. For the test, the cells were first permeabilized with EtOH; during this procedure the low molecular weight DNA inside apoptotic cells leaks out and is removed from the sample during subsequent washing step. The high molecular weight DNA retained in the cells was stained with DAPI. Staurosporine (1 μM) was used as a positive control.

Statistical analysis: For each of the test compounds all the assays were carried out in triplicate. The results were expressed as the mean ± standard error of mean (SEM). After testing the normal distribution of data with the Kolmogorov-Smirnov test, statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by *post-hoc* analysis (Scheffé test) for pair-wise comparisons; the level of significance was set at $p \leq 0.05$. Remaining 4NQO-induced genotoxic activity (RGA%) and genotoxic inhibition rate (GIR%) were calculated according to the following formulas:

$$RGA(\%) = \frac{A-C}{B-C} \times 100 \qquad GIR(\%) = \left[1 - \left(\frac{RGA(\%)}{100}\right)\right] \times 100$$

where A corresponds to the extent of DNA damage observed in cells subjected to the antigenotoxic treatment (*i.e.*, co- or pre-exposure to the test compound plus 4NQO), B corresponds to DNA damage observed in 4NQO exposed cells and C corresponds to DNA damage extent in the negative control.

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