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# Resveratrol and its methoxy-derivatives as modulators of DNA damage induced by ionising radiation

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## Abstract

Various naturally occurring stilbene-like compounds that are related to resveratrol (RSV) possess some of the beneficial effects of the parent molecule and provide even further benefits. Therefore, a series of methoxylated analogues of RSV were prepared with the aim of increasing antitumour and proapoptotic activity. In a previous article, we studied two methoxy-derivatives, pterostilbene (PTERO) and trimethoxystilbene (TRIMETHOXY), in which the first was formed by the substitution of two hydroxyl groups with two methoxy groups (*trans*-3,5-dimethoxy-4'-hydroxystilbene) and the second was formed by the replacement of all three OH groups with methoxy groups (*trans*-3,5,4'-trimethoxystilbene). Both methoxy-derivatives showed stronger antioxidant activity when compared with RSV. In the present article, we focused on the analysis of the ability of RSV and its two methoxylated derivatives to protect proliferating non-tumoural cells from the damage induced by ionising radiation (IR). First we showed that the methoxy derivatives, contrary to their parental compound, are unable to affect topoisomerase enzyme and consequently are not clastogenic *per se*. Second we showed that both PTERO and TRIMETHOXY more efficiently reduce the chromosome damage induced by IR. Furthermore, TRIMETHOXY, but not PTERO, causes a delay in cell proliferation, particularly in mitosis progression increasing the number of cells in metaphase at the expense of prophases and ana/telophases.

## Introduction

Resveratrol (3,4',5-trihydroxystilbene) (RSV) is a well-known natural polyphenol produced by a wide variety of plant species; its synthesis is induced by exposure to microbial infections, ultraviolet radiation and ozone. Many of the proven benefits from RSV are attributed to its antioxidant properties even if many other important functions have been considered to explain its cardioprotective, immunomodulatory, anti-inflammatory and chemopreventive activities (1). Recently, Leone *et al.* (2) reported that RSV impairs Topoisomerase II in human glioma cells. Topoisomerase II is the primary target for some of the most active drugs currently in use for the treatment of

human cancers. Through *in silico* docking simulations the authors demonstrated that RSV polar groups allow this molecule to establish non-covalent cross-linking interactions with both Topoisomerase and DNA at the binding interface between the two macromolecules. Furthermore, Basso *et al.* (3) showed that RSV induces chromosomal damage and malsegregation in mammalian cycling cells.

However, one of the problems regarding the use of RSV as a diet chemopreventive agent is its low oral bioavailability and rapid metabolism. There are several natural stilbenoids that are structurally similar to RSV and also related to its potential metabolism. They may possess some of the beneficial effects of the parent molecule and

provide even greater benefits (4). Studies of these derivatives have provided the basis for the development by selective modification of the stilbene scaffold of novel RSV analogues with more potent antitumour activity or other properties (5). An analysis of the structure of RSV has found that the substitution of the hydroxyl groups with methoxy groups significantly increases the bioavailability through increased intestinal absorption and enhanced hepatic stability (6). Therefore, different methoxylated analogues of RSV have been prepared with the aim of obtaining new agents for cancer chemoprevention.

In a previous article, our group studied two of these derivatives, pterostilbene (PTERO) and trimethoxystilbene (TRIMETHOXY) (7). The first derivative is formed by the substitution of two hydroxyl groups with two methoxyl groups (*trans*-3,5-dimethoxy-4'-hydroxystilbene), and in the second derivative all three OH groups (*trans*-3,5,4'-trimethoxystilbene) have been replaced with methoxy groups (8). PTERO is present in several plant species and has been studied most extensively, while TRIMETHOXY, either natural or synthetic, has not been thoroughly investigated. However, for both molecules several interesting biological activities have been reported in the literature, mostly in the field of chemoprevention (5).

Regarding the antioxidant potential, PTERO displays radical scavenging activity similar to RSV (9); in contrast Stivala *et al.* (10) reported that RSV exerts stronger antioxidant effects than TRIMETHOXY. Our group measured the antioxidant properties of RSV and its methoxy-derivatives, PTERO and TRIMETHOXY, through experimental strategies that enabled us to measure the direct activity of scavenging oxidant species and the protective effect against DNA damage that is induced by exogenously oxidative stress in mammalian cells *in vitro*. Importantly, we found that the methoxylated derivatives appear to be non-mutagenic and surprisingly that the highest protective activity against oxidative DNA damage was exerted by PTERO, then TRIMETHOXY and lastly RSV (7).

In the present article, we have focused on the ability of RSV and its two methoxylated derivatives in protecting proliferating non-tumoural cells from the damage induced by ionising radiation (IR). This research arises from the need to identify non-toxic compounds potentially acting as radioprotectors among natural products with verified antioxidant activity because IR induces extensive damage to biological macromolecules both directly and by oxidative mechanisms. The use of IR has been rapidly increasing for diagnosis and treatment and it is now indispensable in virtually every branch of medicine. Radiation is particularly useful as a component of therapy for a wide range of malignant conditions (11). Since the aim of radiotherapy is to destroy cancer cells with as little damage as possible to normal ones, the role of radioprotective compounds is very important (11).

In this study, we demonstrate the protective activity of PTERO and TRIMETHOXY against radiation-induced chromosome damage in Chinese Hamster Ovary (CHO) cells. We also confirm the anti-topoisomerase II $\alpha$  (TOPOII) activity of RSV and show that its derivatives do not inhibit TOPOII. Furthermore, the ability of RSV derivatives in modulating cell cycle and mitosis progression is discussed.

## Materials and methods

### Reagents and cell culture

Resveratrol (Sigma–Aldrich), pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene) (Sigma–Aldrich) and TRIMETHOXY (*trans*-3,5,4'-trimethoxystilbene) (Enzo Life Sciences) were dissolved in

DMSO immediately before use and supplied to cell cultures at various concentrations (40, 80 and 120  $\mu$ M). DMSO never exceeded 0.02% in the cultures. CHO cells, routinely cultivated in our laboratory, were maintained in Ham's F-10 medium supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin solution, 1% l-glutamine in a 37°C humidified incubator with 5% CO<sub>2</sub>. All reagents were purchased from Euroclone (Milan, Italy).

### Cytotoxicity assay and cytokinesis-block micronucleus assay

Cells were incubated with the different molecules for 6 hrs and analysed immediately after the end of treatment or after 22 h of recovery in fresh medium. The cells were detached with the trypsin/EDTA solution and 90  $\mu$ l of cellular suspension were mixed with 10  $\mu$ l of trypan blue for 3 min. Cells pipetted on a glass slide were analysed under a phase-contrast microscope, for calculating the proportion of not stained (vital) and blue stained (dead) cells.

About  $3 \times 10^5$  cells were seeded in 60-mm Petri dish the day before the experiment; then they were treated for 6 h with different concentrations of the molecules. At the end of the treatment the cells were irradiated with 1 Gy X rays generated by Gilardoni apparatus (operating at 180 Kv, 8 mA, 3 mm Al and delivering 0.5 Gy/min). After irradiation, a recovery time was carried out in fresh medium additioned with 2  $\mu$ g/ml cytochalasin B (Sigma, St Louis, MO, USA) for 22 or 26 h in order to obtain binucleated cells (BNs). Cells were fixed in cold methanol for 10 min, allowed to air dry and stained with the conventional Giemsa method. For each experimental point, at least 1000 BNs were analysed and those with one or more micronuclei (MN) were recorded. The results are expressed as total MN on 1000 BN (MN%). Cell proliferation was evaluated through the nuclear division index (NDI) according to the formula:  $NDI = (1 \times M1 + 2 \times M2 + 3 \times M3 + 4 \times M4)/N$ , where M1 through M4 represent the number of cells with one to four nuclei and N is the total number of cells scored.

### Docking simulations

Docking simulations of RSV, PTERO and TRIMETHOXY onto Type II topoisomerase-DNA complex (PDB ID: 4FM9 (12)); were carried out using the docking program Autodock Vina (13) with a search space (docking grid) that included the whole complex, in order to carry out 'blind' predictions of the small molecules binding sites. RSV spatial coordinates were taken from the Cambridge Crystallographic Data Centre [CCDC code 259251 (14)]. PTERO and TRIMETHOXY spatial coordinates were obtained from Dr Francesco Caruso (7). The docking grid spacing was set to 1 Å per grid unit and the exhaustiveness parameter was increased from the default value of 8 to 24, as suggested by Autodock Vina developers for grid sizes larger than 27 000 Å<sup>3</sup>, which is the case for topoisomerase II simulations.

### Topoisomerase II decatenation assay

Topoisomerase II $\alpha$  activity was determined by assessing the ability of purified topoisomerase II $\alpha$  to decatenate kinetoplast kDNA in the presence of RSV, PTERO and TRIMETHOXY. Decatenation assay was performed with 296 ng kinetoplast DNA (kDNA) (TopoGEN Inc.) in a 20  $\mu$ l reaction containing 50 mM Tris–HCl, pH 8.0, 120 mM KCl, 10 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 2 mM ATP with or without 120  $\mu$ M of RSV and its derivatives, 30  $\mu$ g/ml bovine serum albumin and 3 U of human purified Topo II $\alpha$  (TopoGEN Inc.). Reactions were incubated at 37°C for 1 h and stopped by adding 5  $\mu$ l

stop buffer (5% N-lauroylsarcosine, 0.125% bromphenol blue and 25% glycerol). Samples were loaded directly onto a 1% agarose gel containing ethidium bromide (0.5 µg/ml) and the inhibition of decatenation was determined by the loss of migration of high molecular kDNA catenated which remained in the well. Decatenated and linear kDNA markers (TopoGEN Inc.) were loaded as reference.

### Mitosis progression assay

After 1 h treatment with PTERO or TRIMETHOXY, cells were fixed two times in methanol/glacial acetic acid 3:1 mixture for 10 min and stained with conventional Giemsa method. For each experimental point, at least 200 mitosis were analysed and the frequency of prophase, metaphases and ana/telophases was scored (3). Mitotic phases were judged as follows: prophase = chromatin condenses and chromosomes begin to be visible, prometa/metaphase = chromosomes are visible at their most condensed stage, ana/telophase = sister chromatids move to opposite ends of the cell and then are grouped into daughter nuclei where chromatin unwinds.

### Flow cytometry for cell cycle analysis

For studying the effect of TRIMETHOXY treatment on cell distribution in the different phases, cells were treated for 6 h with the molecule and then with BrdU (45 µM) for 30 min. BrdU incorporation allows to distinguish S phase cells from G1 and G2 phase ones. DNA content and BrdU incorporation were determined by flow cytometry.

Flow cytometric analysis was carried out using on an Epics XL apparatus (Beckman Coulter) equipped with a 15 mW Innova 90 Coherent laser with a 488 nm wavelength excitation light. At the end of treatment cells were fixed in a 1:1 methanol: PBS mixture and DNA was denaturated in 3 N HCl for 45 min. After neutralising in 0.1 M Na2B4O, cells were incubated with an anti-BrdU antibody (1:50, Dako) for 1 h at room temperature, washed in PBS + 0.5% Tween 20, and then incubated for 45 min with Alexa Fluor 488-conjugated secondary antibody (1:600, Molecular Probe).

After extensive washing, cells were resuspended in PBS containing 20 µg/ml propidium iodide and analysed for their DNA content (red fluorescence at 620 nm) and BrdU incorporation (green fluorescence at 510 nm). Ten thousand events were collected from each sample. Biparametric plots of DNA content and BrdU incorporation were analysed using WinMDI 2.9 software.

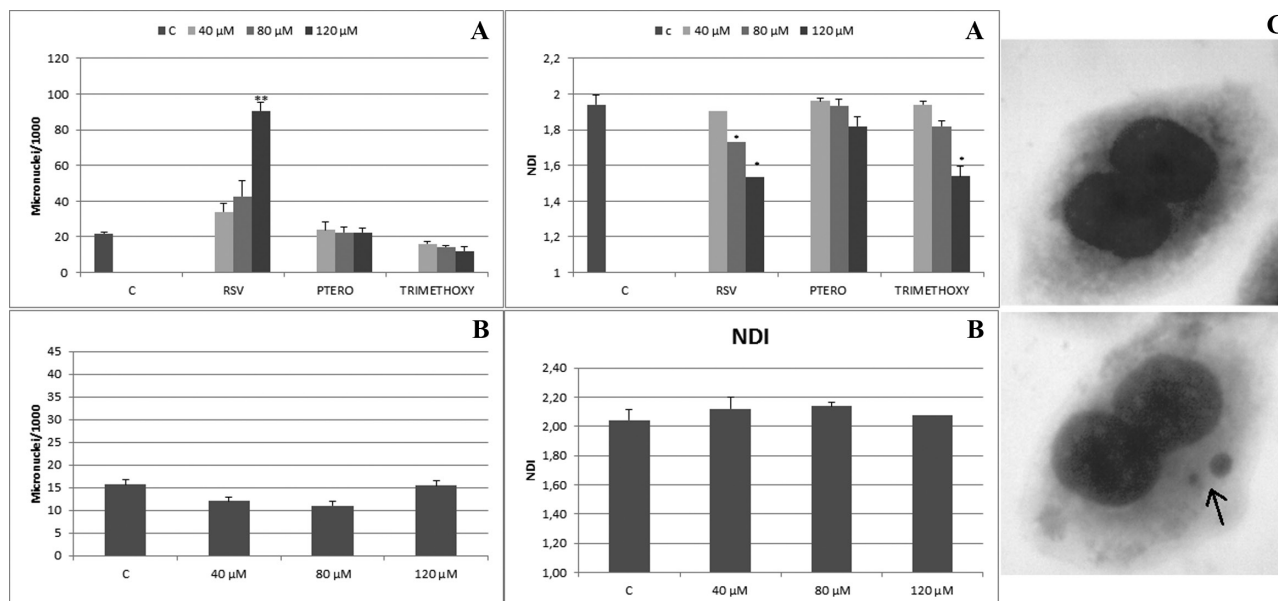
### Statistical analysis

All data were obtained through at least three independent experiments and expressed as means ± standard deviations (SD). The non-parametric Kruskal–Wallis test (significance taken as  $P < 0.05$ ) was applied to compare exposed cells to control ones in genetic damage assays. Statistical analysis of data was performed using Graph Pad software InStat version 3.02 (Graph Pad Software, San Diego, CA).

## Results

### Genotoxicity of RSV and its methoxy-derivatives

First, we examined the genotoxic effects of RSV, PTERO and TRIMETHOXY treatment on CHO cells as evaluated by chromosomal damage induction. We started from our previous observation (3) that RSV *per se* is able to induce a dose-dependent increase in chromosome damage coupled to a reduction of cell proliferation. In these experiments, we utilised a cytokinesis-block micronucleus (CBMN) assay that is a very reliable, thoroughly validated and standardised technique to measure chromosome breaks. Data shown in Figure 1A confirm the clastogenic activity of the highest dose of RSV, while the derivatives do not induce any increase of MN. A 6 h treatment with the highest dose of TRIMETHOXY causes a significant reduction of NDI ( $P < 0.05$ ), thus we extended cytochalasin treatment from 22 to 26 h, obtaining a recovery of NDI after TRIMETHOXY treatment without any variation in MN induction (Figure 1B). At the same time, we also measured the effect of the treatments on cell viability through trypan blue exclusion. The dead cells never exceeded 10% with the highest dose of the drug treatments (from 1% in untreated



**Figure 1.** CHO cells were treated with different concentrations of RSV, PTERO or TRIMETHOXY for 6 h. MN induction and NDI were assessed by CBMN assay after 24 h (A) of cytochalasin treatment. In a different set of experiments, CHO cells were treated with three concentrations of TRIMETHOXY for 6 h and cytochalasin treatment lasted 26 h (B). The images in (C) show a BN without or with a micronucleus (arrow). Data are represented as mean ± SEM of at least three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  at Kruskal–Wallis test compared to untreated cells (C).

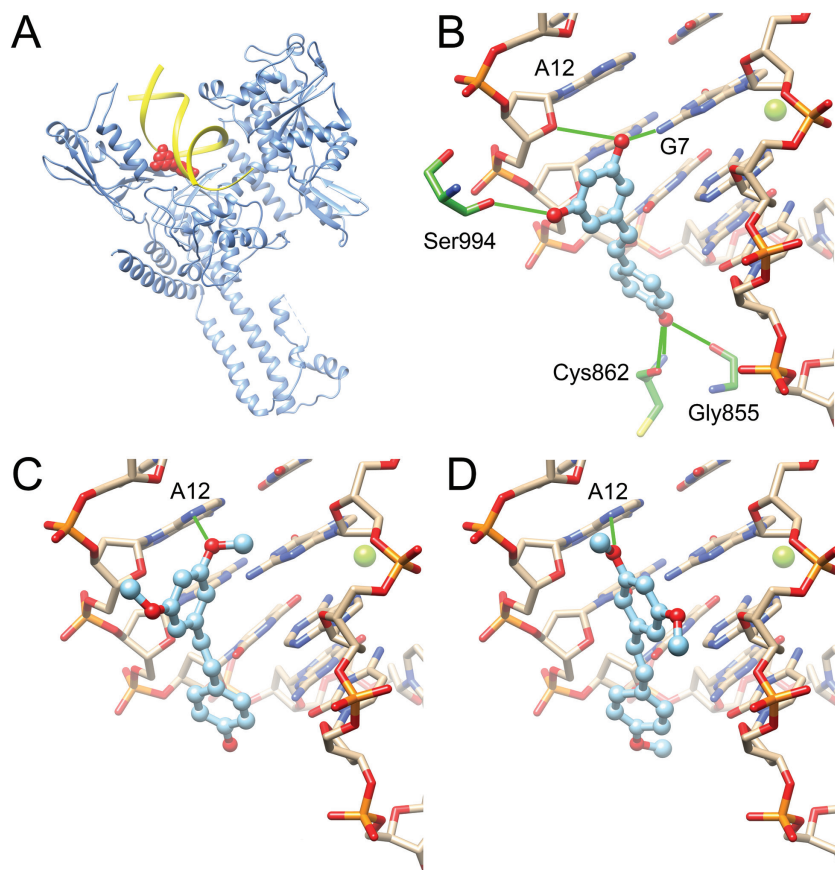
cells to 7%, 8% and 10% for PTERO-, TRIMETHOXY- and RSV-treated cells, respectively). On the whole these data suggest that RSV methoxy-derivatives are not clastogenic.

### Molecular interactions of RSV, PTERO and TRIMETHOXY with DNA and TOPOII

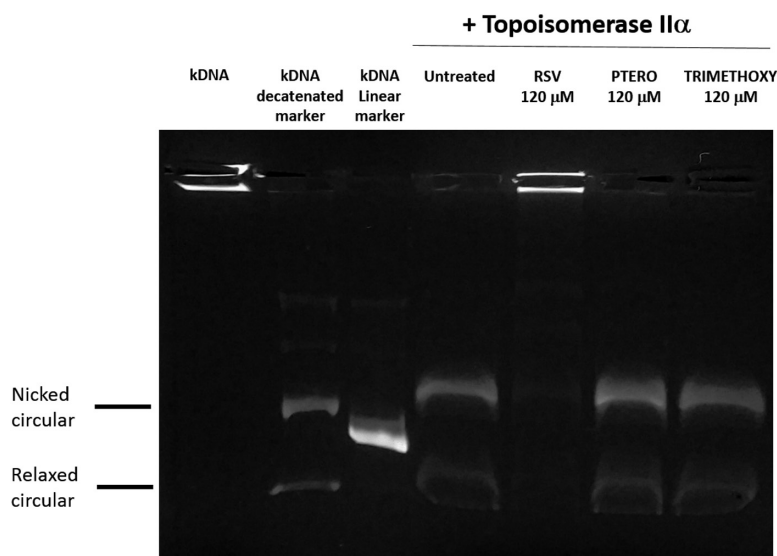
The absence of any chromosome damage induced by PTERO and TRIMETHOXY treatment suggested that these two RSV derivatives do not interfere with TOPOII. Therefore, the possibility of a direct interaction of RSV and its derivatives with TOPOII was investigated *in silico* using the flexible docking protocol implemented in Autodock Vina (13). Figure 2 shows the lowest energy complexes obtained for each of the three compounds. Interestingly, in the case of RSV the complex is very similar to that obtained in a previous study from our lab (2) employing the rigid-body docking protocol implemented in PatchDock (15). In this new study, we confirmed our previous prediction that RSV binds at the TOPOII-DNA interface establishing several hydrogen bonds with both the protein groups and DNA bases and sugars, while no carbohydrate-aromatic stacking interactions with DNA deoxyribose moiety are observed (Figure 2). In detail, one of the hydroxyl groups of RSV (O1) forms hydrogen bonds with the Cys862 backbone amide and carbonyl groups and with the backbone carbonyl group of Gly855. Another RSV hydroxyl group (O3) is instead bound to DNA by hydrogen bonds with the N2 atom of the Guanosine 7 and with

the deoxyribose moiety O4' atom of Adenosine 12. Docking simulations indicate that PTERO and TRIMETHOXY can bind in the same region of the TOPOII-DNA interface. However, the number of interactions that these compounds establish with TOPOII and DNA are much lower (Figure 2), partly due to a slightly different binding mode and to the substitution of two and three hydroxyl group, respectively, with methoxy groups that can act only as hydrogen bond acceptors and not as donors. In detail, both PTERO and TRIMETHOXY form only one hydrogen bond with adenine moiety N3 atom of Adenosine 12. These differences are reflected in a higher calculated binding energy of the two latter compounds for the TOPOII-DNA complex ( $-7.0$  and  $-7.1$  kcal/mol for PTERO and TRIMETHOXY, respectively) with respect to RSV ( $-7.9$  kcal/mol). Thus, the binding mode observed in docking simulations indicates that RSV could increase the stability of the TOPOII/DNA complex by 'cross-linking' the two macromolecules through the interactions described above. Observations indicate that these interactions occur to a lesser extent for PTERO and TRIMETHOXY.

To verify the different ability of RSV and its derivatives in interfering with TOPOII, we monitored their capacity to inhibit the ability of human recombinant TOPO II to decatenate kinetoplast DNA (kDNA). As shown in Figure 3, when TOPOII is added, kDNA is cleaved and fragments are electrophoresed; when  $120 \mu\text{M}$  of RSV is added to the mixture (TOPOII + kDNA), it completely inhibits the decatenation of kDNA. In agreement with the docking simulation,



**Figure 2.** Schematic representation of the complexes between RSV, PTERO and TRIMETHOXY and TopoII obtained by docking simulations. (A) Schematic view of the RSV-TopoII complex showing the location of the RSV and derivatives binding site at the interface between the protein (blu) and DNA (yellow). RSV is shown in spacefill representation (red). (B) Atomic details of the RSV-TopoII complex. (C) Atomic details of the PTERO-TopoII complex. (D) Atomic details of the TRIMETHOXY-TopoII complex. Hydrogen bonds are represented by green lines. Small molecule ligands are represented in ball-and-stick style. See text for details.



**Figure 3.** Topoisomerase II decatenation assay was performed incubating kinetoplast DNA and human purified Topoisomerase II $\alpha$  in absence (untreated) or presence of 120  $\mu$ M of RSV, PTERO or TRIMETHOXY for 1 h. Nicked and relaxed circular DNA represent a topoisomerase II decatenation products. kDNA catenated is retained in the well due to its large size.

both PTERO and TRIMETHOXY, at the same dose used with RSV, did not inhibit TOPOII activity, thus the kinetoplast DNA is completely decatenated.

#### The effect of PTERO and TRIMETHOXY on the cell cycle progression

Since the reduction of NDI suggested a delaying effect on the cell cycle progression induced by TRIMETHOXY treatment (but not by RSV and PTERO), we performed the analysis of cell cycle progression through the incorporation of BrdUrd. **Figure 4** shows the distribution (dot plots) of cells in the different cell cycle phases (A) and the relative proportions in G<sub>1</sub>, S and G<sub>2</sub>/M (B). TRIMETHOXY induces a reduction in S phase cells (from 52.5% in the untreated sample to 48.8% at the highest dose of TRIMETHOXY) with a corresponding increase in G<sub>2</sub>/M cells (from 4.1% in control to 8% at the highest dose of TRIMETHOXY). However, this difference is not statistically significant.

To determine whether the observed increase in G<sub>2</sub>/M cells could be the consequence of a delay that occurs particularly during mitosis progression we monitored the capacity of PTERO and TRIMETHOXY treatment to modify the distribution of the cells in the different phases of mitosis. In **Figure 5**, we show that PTERO treatment at 120  $\mu$ M causes a slight non-significant increase in the percentage of pro-metaphases and metaphases with a concomitant slight decrease in ana-telophase cells in comparison to an untreated sample. Regarding TRIMETHOXY treatment the increase in metaphase cells is very high at all doses (80% compared with 30% in the untreated sample  $P < 0.001$ ) with a concomitant reduction of pro-phases and ana/telophases. At the highest doses the ana/telophases are just 2% ( $P < 0.001$  compared to untreated sample). On the whole the data show that TRIMETHOXY, but not PTERO, causes a delay in mitosis progression resulting in the accumulation of cells in metaphase.

#### Effect of RSV and its methoxy-derivatives on MN induced by X rays

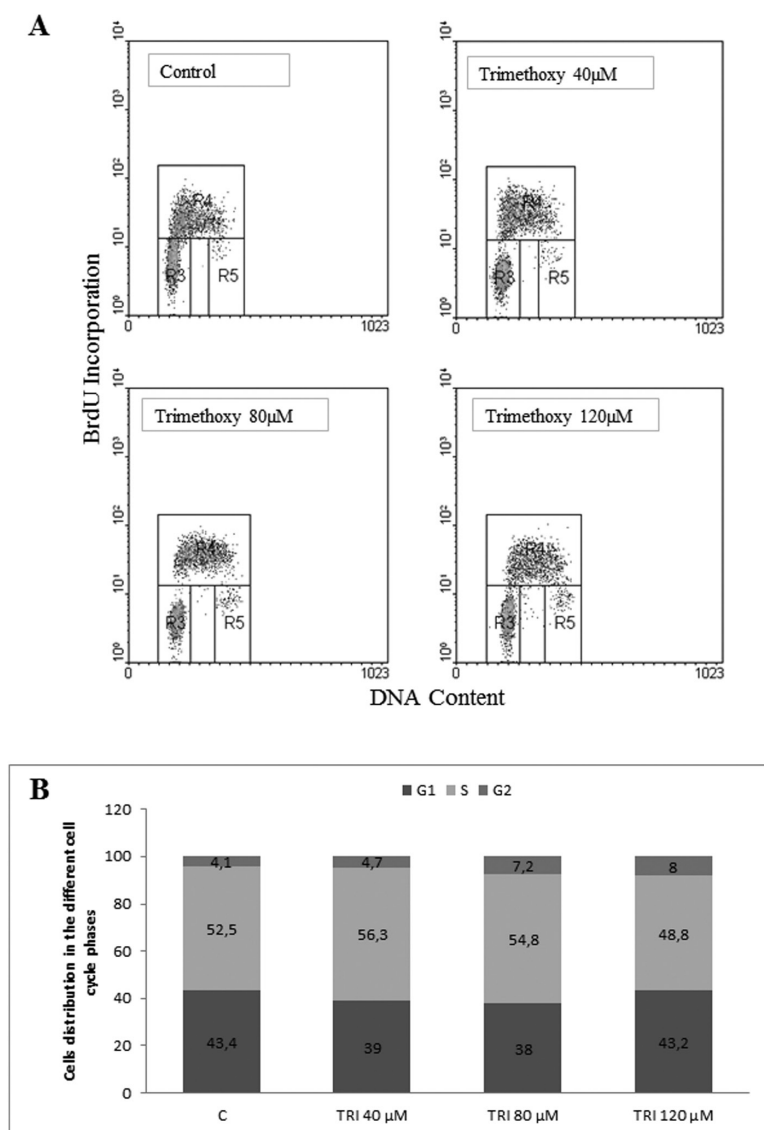
In the second part of our experiments we analysed the ability of the three molecules to modulate the chromosome damage induced

by IR. **Figure 6A** shows the data on MN induction and NDI. As expected, 1 Gy X ray treatment causes a very significant induction of MN ( $P < 0.001$ ) compared with the control with a slight non-significant reduction in NDI. In the combined treatment RSV + IR we did not observe any change in MN induction compared with IR-treated cells. Conversely, we always observed a significant ( $P < 0.05$ ) reduction of MN in the combined treatments PTERO + IR when compared with IR single treatment; TRIMETHOXY in combination with IR appears to be much more effective in reducing the clastogenic effect of IR showing a very significant reduction at all doses ( $P < 0.01$  and  $P < 0.001$ ). The combined treatments with the highest dose of TRIMETHOXY resulted in a significant ( $P < 0.05$ ) reduction in NDI, thus we extended cytochalasin treatment obtaining a recovery of NDI. Similar to the first set of experiments, the reduction of MN (from 556‰ in IR treated sample to 337, 258 and 193‰ in combined treatments with the different doses of TRIMETHOXY) persists significantly ( $P < 0.01$  and  $P < 0.001$ ) in this set of experiments (**Figure 6B**).

On the whole these results indicate a substantial inability of RSV in modulating IR induced damage; conversely, PTERO, and especially TRIMETHOXY, exert strong protective activity against the clastogenic action of IR.

#### Discussion

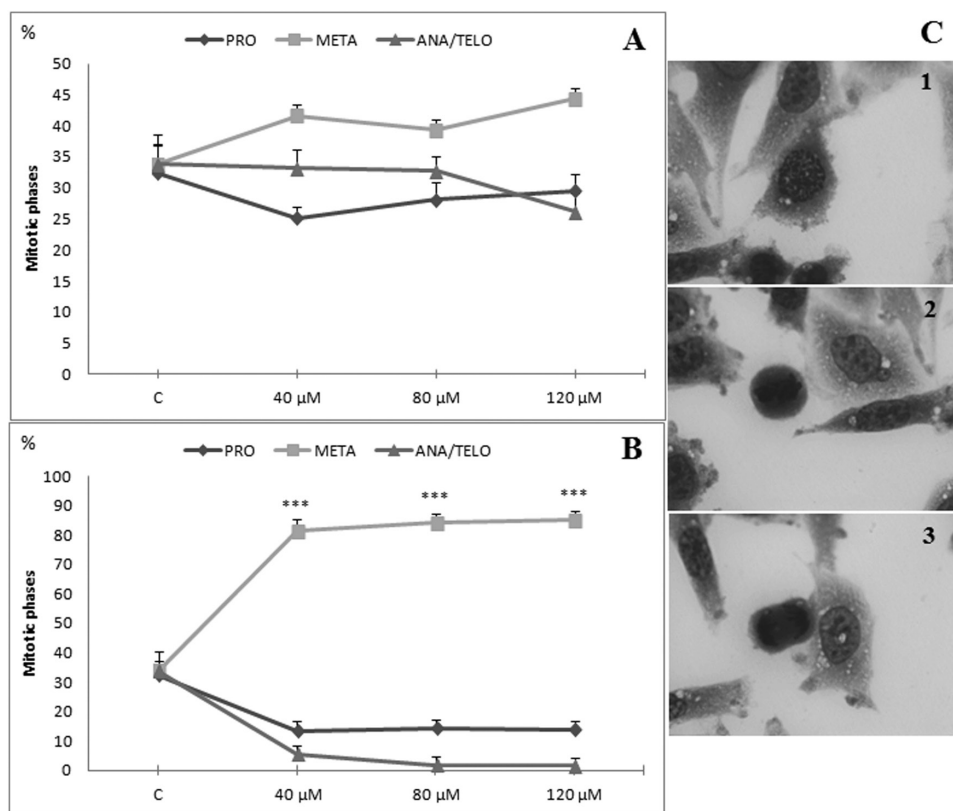
In this article, we investigated the potential protective activity of two methylated derivatives of RSV against exogenously induced DNA damage. The interest in these molecules arises from two important aspects regarding RSV, namely its limited potential use due to rapid metabolism and poor bioavailability (16) and the clastogenic effect of RSV treatment potentially due to its capacity in interfering with topoisomerases (2) and histone deacetylases (17). For these reasons the analysis of structurally modified RSV analogues may lead to the identification of chemicals showing features and activities capable of counteracting some of the mentioned limitations in using RSV as a chemopreventive agent. In particular, plasma levels of the dimethoxy derivative PTERO are much higher than those of equimolar doses of RSV, regardless of the dose or route of administration (18).



**Figure 4.** CHO cells were treated for 6 h with three concentrations of TRIMETHOXY. A 30' pulse of BrdU was added at the end of treatment. Immediately after the end of pulse, a flow cytometric analysis was performed (A) to determine the distribution of cells in G1/S/G2 phases of cell cycle (B).

Our first aim was to analyse the potential cytotoxicity of two methylated derivatives, PTERO and TRIMETHOXY, compared with RSV in normal mammalian cells. While confirming the clastogenic activity of RSV we showed that at the same doses both PTERO and TRIMETHOXY do not cause any chromosome damage; furthermore, the two molecules are unable to interfere with topoisomerase II $\alpha$ . In our opinion, this latter result is particularly important because through using a more accurate docking simulation we confirmed the previously demonstrated ability of RSV polar groups to establish non-covalent cross-linking interactions with both TOPOII and DNA (2,19). However, overall we showed for the first time that the substitution of hydroxyl groups with methoxy ones renders the interaction between the two RSV derivatives with DNA and TOPOII much weaker due to the reduced numbers of hydrogen bonds between the methoxy derivatives, TOPOII and DNA. This behaviour would explain the inability of methoxy-derivatives to inhibit TOPOII activity and consequently to form potentially clastogenic ternary complexes (derivatives-DNA-TOPOII).

The second important aspect is the ability of TRIMETHOXY, but not PTERO, to induce a delay in cell proliferation as indicated by NDI and cell cycle analysis, which is mainly dependent on a delay of mitosis progression. In particular, our data showed that TRIMETHOXY treatment induces a strong increase in pro-metaphases and metaphases with a concomitant decrease in both pro-metaphases and ana/telophases. These results are in agreement with the literature data showing that TRIMETHOXY acts as an inhibitor of tubulin polymerisation in cancer cells (20,21). Belleri *et al.* (22) also showed the microtubule destabilising activity of TRIMETHOXY, but not RSV, in endothelial cells. Through a computational docking approach, Mazué *et al.* (23) have shown that TRIMETHOXY integrates well within the colchicine-binding site of tubulin (the hydrophobic pocket) that is responsible for mitosis inhibition and polyploidy induction. Certainly this behaviour renders this RSV derivative applicable as a potential candidate for fighting cancer. While it has been demonstrated by our and other groups that RSV delays cancer cell proliferation during the S phase (19,23,24),



**Figure 5.** CHO cells were treated for 1 h with three concentrations of PTERO (A) or TRIMETHOXY (B) and the presence of prophases, prometa/metaphases and ana/telophases was evaluated. The images show a prophase (C1), a prometa/metaphase (C2), an ana/ telophase (C3). \*\*\* $P < 0.001$  at Kruskal–Wallis test compared to untreated cells (C).

here we reported that TRIMETHOXY drastically inhibits mitosis progression, thus showing a different way of delaying cell cycle progression.

Last we examined the potential protective activity of RSV and its methoxy-derivatives against irradiation induced DNA damage. This interest arises from the recognition of the need of molecules that are able to protect normal cells during a radiodiagnostic/radiotherapeutic procedure. A wide variety of natural compounds tested for their activity has shown radio-protective properties coupled with an unacceptable toxicity that limits their clinical utility.

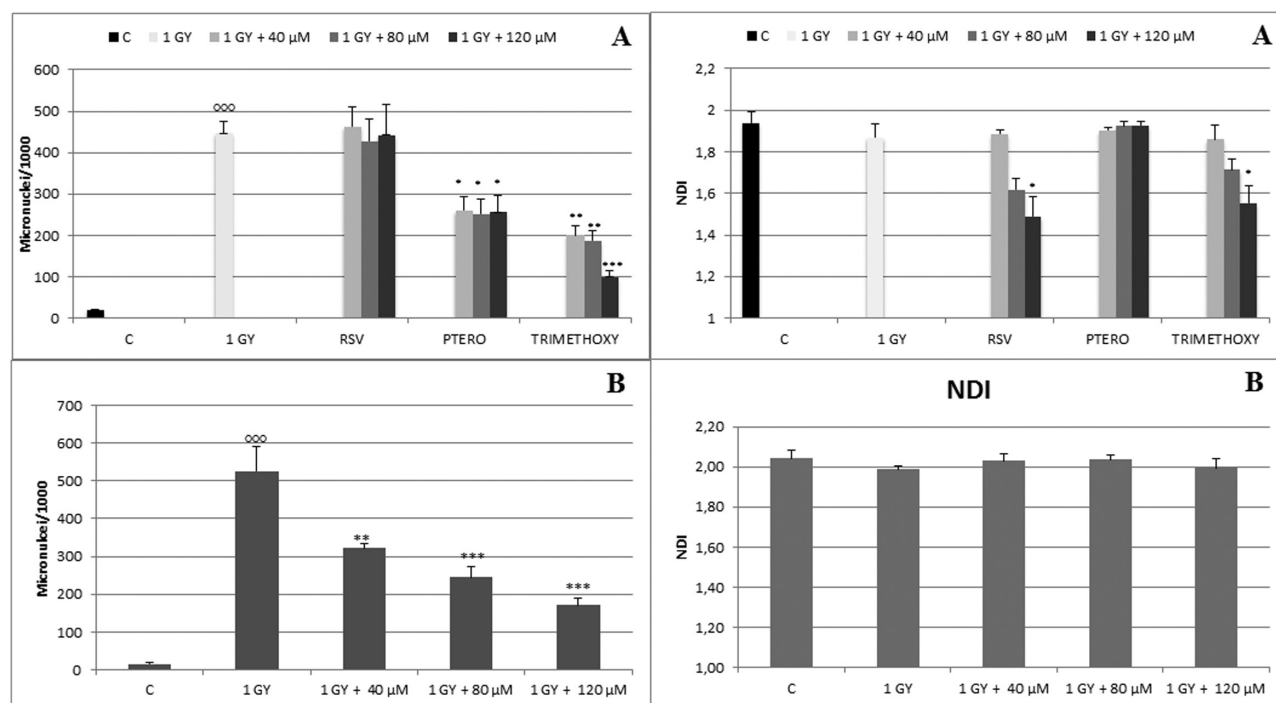
Here, we showed a clear protective activity exerted by PTERO and mostly by TRIMETHOXY. Pre-treatment of cells with PTERO significantly reduced IR-induced MN up to 57%, while TRIMETHOXY pre-treatment reduced IR induced chromosome damage in a dose dependent manner, reaching a 65% of reduction at the highest dose without affecting the NDI. These data are very surprising because comparable results are uncommon with natural compounds except for a small number of molecules (25,26). Recently, Cinkilic *et al.* (27) have shown that cinnamic acid, a phenolic compound, produces efficient radioprotection of human lymphocytes against the damaging effects of X-rays without showing any cytotoxicity.

Regarding RSV, we showed that it is unable to modulate IR-induced damage. This result was expected considering that RSV *per se* induces chromosome damage due to its antitopoisomerase activity.

The three molecules we used are documented as antioxidants even if their activity is dependent on their concentrations and the substitution of hydroxyl groups with methoxy groups seem to confer a stronger antioxidant activity to PTERO and TRIMETHOXY than

RSV (7). However, at the moment it is difficult to attribute the protective effect exerted by the methoxy-derivatives against IR-induced damage only to their antioxidant activity; rather, several pieces of evidence demonstrate that the introduction of methoxy groups at the stilbene motif of RSV is important to confer a different mode of action to this class of compounds. Simoni *et al.* (28) have shown a strong pro-apoptotic activity exerted by TRIMETHOXY at very low concentrations. More recently, Aldawsari and Velazquez-Martinez (29) have reported a summary of literature data demonstrating that TRIMETHOXY is capable of inducing cycle arrest and apoptosis by different mechanisms of action than those observed for RSV, and in some cases, with an improved potency and efficacy. On the other hand, also PTERO seems to exert pro-apoptotic activity against different cancer cells both inhibiting cell proliferating factors and inducing mitochondrial apoptotic signals (30). Nonetheless, literature data agree on the important biomedical applications for PTERO and TRIMETHOXY in cancer prevention and treatment (5,31).

In conclusion, in this study we analysed the potential use of RSV and its methoxy-derivatives as radioprotective compounds. We showed the absence of genotoxicity of PTERO and TRIMETHOXY compared with RSV. To our knowledge this is the first report where this aspect is analysed from the point of view of Topoisomerase II inhibition. Furthermore, the radio-protective activity exerted by the two derivatives is clearly demonstrated compared with the substantial inability of RSV. In our opinion, this result is particularly interesting as it confirms that the substitution of hydroxyl groups with methoxy groups may change cellular/molecular targets thus deeply modifying the behaviour of the derivatives. Clearly, these data need



**Figure 6.** CHO cells were treated with different concentrations of RSV, PTERO or TRIMETHOXY for 6h. At the end of treatment the cells were irradiated with 1 Gy of X rays. MN induction and NDI were assessed by CBMN assay after 24h of cytochalasin treatment (A). In a different set of experiments, CHO cells were treated with three concentrations of TRIMETHOXY for 6h, then irradiated. Cytochalasin treatment lasted 26h (B).  $^{\circ\circ\circ}P < 0.001$  at Kruskal–Wallis test compared to untreated cells.  $*P < 0.05$ ,  $**P < 0.001$  and  $***P < 0.001$  at Kruskal–Wallis test compared to irradiated cells.

to be confirmed in normal human cells but the evidence thus far is very encouraging.

## Highlights

1. Micronuclei induction by resveratrol, pterostilbene and trimethoxystilbene treatment.
2. Methoxy-derivatives do not inhibit topoisomerase II $\alpha$  activity.
3. Docking simulation of DNA/molecules/topoisomerase interaction.
4. Methoxy-derivatives protect DNA against radiation-induced damage.

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Conflict of interest statement: None declared.

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