SCUOLA DOTTORALE IN BIOLOGIA

SEZIONE SCIENZE BIOMOLECOLARI E CELLULARI

XXV CICLO

Chemopreventive and Antitumoral Properties of Resveratrol

Proprietà Chemiopreventive ed Antitumorali del Resveratrolo

Dottorando: Emiliano Basso

A.A. 2012/2013

Docente Guida: Prof.ssa Renata Cozzi

Coordinatore: Prof. Paolo Mariottini

INDEX

Summary 4
1 INTRODUCTION 10
1.1 The Cancer Chemoprevention 10
1.2 Resveratrol in Chemoprevention 12
1.3 Resveratrol in Cell Proliferation and Apoptosis 15
1.4 Resveratrol and DNA 16
1.4.1 RESV and DNA damage in non-cancer cells 17
1.4.2 RESV and DNA damage in cancer cells 18
1.5 DNA Topoisomerases 20
1.6 REFERENCES 23
2 AIM OF THE RESEARCH 28
3 PUBLICATIONS 29
3.1 Leone, S., Cornetta, T., **Basso, E**. and Cozzi, R. (2010) *Resveratrol induces DNA double-strand breaks through human topoisomerase II*. Cancer Lett, 295, 167-72 30
3.2 Leone, S., **Basso, E**., Polticelli, F. and Cozzi, R. (2012) *Resveratrol acts as a topoisomerase II poison in human glioma cells*. Int J Cancer, 131, E173-8. 36
3.3 **Basso, E**., Fiore, M., Leone, S., Degrassi, F. and Cozzi, R. (2012) *Effects of Resveratrol on Topoisomerase II-α activity: induction of micronuclei and inhibition of chromosome egregation in CHO-K1 cells*. Mutagenesis (in press). 42
4 DISCUSSION 48
4.1 REFERENCES 54

Summary

A great interest has grown during last decades about the use of natural dietary compounds to inhibit the onset of several human diseases, among them cancer. The study of preventive effects of phytochemicals respect to cancer incidence is called chemoprevention.
A chemopreventive agent must be able to act in one or more steps of carcinogenesis blocking or suppressing the onset and progression of cancer. The stilbene resveratrol (3,5,4'-*trans*-hydroxystilbene) (RESV) has attracted a great interest as it could exert many potential beneficial effects in human health.
In my host lab it has been previously described the capacity of RESV to re-establish Gap Junction Intercellular Communications (GJIC) in glioma cells, with a concomitant delay in the S-phase of cell cycle. Gliomas are highly malignant tumors owning a strong resistance to conventional therapies and the cells lost GJIC in the promotion/progression steps of carcinogenesis. So we wondered which kind of regulatory factors could be spread across cells after RESV treatment.
During my PhD project we investigated the antiproliferative activity exerted by RESV and we demonstrated that its chemopreventive role could be in part explained through the interaction with Topoisomerase II-α (TOPO2) activity.
In the first part we analysed the effects of RESV on cell cycle progression and DNA damage induction in glioma cells. At first we performed a cytofluorimetric biparametric assay in order to follow the phosphorylation of the histone H2AX (γ-H2AX) during cell cycle. γ-H2AX is a variant form of the histone H2A that is phosphorylated in response to DNA double strand breaks (DSBs) induction. For this reason it is considered a hallmark of DSBs. We found a delay in the S-phase with a concomitant induction of γ-H2AX. These data have let us to suppose a putative interaction between RESV and TOPO2.
TOPO2 is an enzyme mainly expressed in proliferating cells, from the S-phase, where it is involved in DNA supercoiling removal, to the G2/M phase, where it decatenates intertwined chromatids and drives a proper chromosome segregation. Through an *in vitro* decatenation test we demonstrated that RESV can affect TOPO2 activity.
The inhibition of TOPO2 activity can occurs through two ways: the catalytic inhibition, that inactivates the enzyme without inducing DNA DSBs and the TOPO2-poisoning, that through the stabilization of the complex DNA-TOPO2, namely the "cleavage complex", causes DNA DSBs induction.

In the second part of my PhD project we investigated whether RESV could act as a TOPO2-poison in glioma cells. We performed a Docking Simulation and we observed *in silico* that RESV was able to act as bridge between DNA and TOPO2 establishing several hydrogen bonds with both molecules. Starting from this preliminary data we verified the stabilization of cleavage complexes by RESV in glioma cells too, through the *In Vivo* Complex of Enzyme Assay (ICE). Then we analysed the consequences of the TOPO2-poisoning in terms of DNA damage induction and DNA damage response signalling pathways activation. We performed the Cytokinesis-Block Micronucleus Assay (CBMN) in order to detect the DNA DSBs induction and we found that RESV caused a significant increase of micronuclei frequency. Afterwards we analysed the activation of factors involved in the DNA damage response pathway, namely ATM/ATR and their kinase effectors Chk2 and Chk1. We found that RESV activates ATM that in turn causes the activation of Chk2. As H2AX is one of the substrates of ATM, we analysed the phosphorylation of H2AX immediately after RESV treatment and 24 hours of recovery time. Our data showed that RESV causes the induction of γ-H2AX that persists until 24 hours. Surprisingly we didn't observe any effect in the activation of ATR and Chk1.

In the last part of my PhD project we focused the attention on the consequences of TOPO2-poisoning in CHO cells, that are normal but proliferating cells. In particular we analysed the effects of RESV treatment respect to chromosome stability, namely DNA damage induction and inhibition of chromosome segregation.

We found an increase of polyploidy (POL) and endoreduplicated (ENDO) cells as a consequence of chromosome segregation inhibition exerted by RESV. Endoreduplication is a phenomenon that consists in a new DNA synthesis round without a cell division. Then we performed a CBMN assay and we found a significant increase of micronuclei frequency as previously shown in glioma cells. Interestingly the co-treatment with the TOPO2 inhibitor EtBr caused a significant reduction in micronuclei frequency, indicating that both RESV and EtBr are competitor for the binding to TOPO2. Moreover a CBMN assay with an anti-kinetocore staining allowed us to find out that RESV caused the production of micronuclei positive for kinetocore indicating the presence of a whole chromosome. So RESV not only induces DSBs but also causes chromosome loss. In order to analyse the effects of the inhibition of chromosome segregation respect to mitotic progression, we analysed the frequency of mitotic figures after RESV treatment. We found an accumulation of cells in the prometa/metaphase stage at the expense of ana/telophase one. The manifestation of chromosome segregation inhibition is the presence of anaphase bridges at the

ana/telophase stage. We found a significant increase of anaphase bridges after treatment with the highest dose of RESV.
On the whole our data confirm the chemopreventive role of RESV in cancer cells and emphasize a new aspect of this compound not investigated in the past. Indeed we showed here for the first time that RESV is able to poisoning TOPO2, inducing in this manner DNA DSBs and activating pathways involved in DNA damage signalling and cell cycle control in cancer cells. Moreover it affects chromosome stability at various levels in CHO cells as a consequence of TOPO2 poisoning. These evidences create a new insight into RESV landscape, supporting the idea that it could be utilised as chemopreventive and/or as an adjuvant of conventional therapies against cancer.

Negli ultimi decenni è emerso un grande interesse circa l'uso di composti naturali presenti nella dieta in grado di inibire l'insorgenza di numerose malattie umane, tra cui il cancro. Con questa prospettiva si è ampiamente sviluppata l'idea della chemioprevenzione, come assunzione di composti naturali bioattivi che agiscano come agenti chemiopreventivi.
Un agente chemiopreventivo deve essere in grado di agire in uno o più stadi della cancerogenesi, bloccando o sopprimendo l'insorgenza e la progressione del cancro. Lo stilbene resveratrolo (3,5,4 '-trans-hydroxystilbene) (RESV) ha suscitato molto interesse in quanto potrebbe esercitare molti potenziali effetti benefici per la salute umana.
Nel laboratorio che mi ha ospitato, era già stata descritta in esperimenti *in vitro* la capacità del RESV di ristabilire le giunzioni intercellulari (Gap Junction Intercellular Communications) in cellule di glioma umano, in concomitanza con l'induzione di ritardo nella progressione della fase S del ciclo cellulare. I gliomi sono tumori particolarmente aggressivi che possiedono una forte resistenza alle terapie convenzionali e le cui cellule perdono le giunzioni nelle fasi di promozione/progressione dello sviluppo tumorale. Da questi risultati è derivata la domanda su quali fattori di regolazione e/o di danno potessero diffondere da una cellula all'altra dopo il ripristino delle GJICs, favorito dal trattamento con RESV.

Durante il mio progetto di dottorato abbiamo valutato l'attività antiproliferativa esercitata dal RESV e abbiamo dimostrato che il suo ruolo chemiopreventivo potrebbe essere in parte spiegato attraverso l'interazione con la topoisomerasi II-α (TOPO2).
Nella prima parte abbiamo analizzato gli effetti del RESV sulla progressione del ciclo cellulare e l'induzione del danno al DNA in cellule di glioma. Dapprima abbiamo eseguito un'analisi citofluorimetrica biparametrica per seguire la fosforilazione dell'istone H2AX (γ-H2AX) durante il ciclo cellulare. γ-H2AX è una forma variante dell'istone H2A che viene fosforilata in seguito alla formazione di rotture a doppio filamento del DNA (DSBs). Per questo motivo viene considerato un marcatore di DSBs. Abbiamo trovato un ritardo nella progressione della fase S con una induzione di γ-H2AX. Questi dati hanno lasciato supporre una probabile interazione tra RESV e TOPO2.
TOPO2 è un enzima principalmente espresso in cellule proliferanti, durante la tarda fase S, dove è coinvolto nella rimozione dei superavvolgimenti del DNA, fino alla fase G2/M, dove decatena i cromatidi e guida una corretta segregazione cromosomica. Abbiamo dimostrato attraverso un test di decatenazione *in vitro* che il RESV influenza l'attività della TOPO2. L'inibizione della TOPO2 può avvenire attraverso due vie: l'inibizione

catalitica, che inattiva l'enzima senza indurre DSBs e l'avvelenamento, che attraverso la stabilizzazione del complesso DNA-TOPO2, cioè il "complesso di taglio" (*cleavage complex*), provoca DSBs.
Nella seconda parte del mio progetto di dottorato abbiamo valutato se il RESV potesse fungere da "veleno" della TOPO2 in cellule di glioma. Abbiamo eseguito una simulazione al computer (Docking molecolare) e abbiamo osservato *in silico* che il RESV era in grado di interagire con entrambe le molecole, fingendo da ponte e stabilendo diversi legami idrogeno. A partire da questi dati preliminari, abbiamo verificato la stabilizzazione dei suddetti complessi nelle cellule di glioma, attraverso l'*In Vivo Complex of Enzyme Assay* (ICE). Quindi abbiamo analizzato le conseguenze dell'avvelenamento della TOPO2 in termini di induzione di danno al DNA e attivazione delle vie di segnalazione del danno. Per questo scopo, abbiamo eseguito il test dei micronuclei, al fine di rilevare l'induzione di DNA DSBs e abbiamo scoperto che il trattamento con RESV causava un aumento significativo della frequenza di micronuclei. Successivamente abbiamo analizzato l'attivazione di fattori coinvolti nella via di risposta al danno al DNA, cioè ATM/ATR e dei loro effettori, le chinasi Chk2 e Chk1, tramite *western blotting*. Abbiamo trovato che il RESV attiva ATM che a sua volta fosforila Chk2. Poiché H2AX è uno dei substrati di ATM, abbiamo analizzato la fosforilazione di H2AX immediatamente dopo il trattamento con RESV e dopo 24 ore di recupero. I nostri dati hanno mostrato che il RESV provoca l'induzione di γ-H2AX che persiste fino a 24 ore. Sorprendentemente non abbiamo osservato alcuna attivazione di ATR e Chk1.
Nell'ultima parte del mio progetto di dottorato abbiamo focalizzato l'attenzione sulle conseguenze dell'avvelenamento della TOPO2 utilizzando come sistema di studio le cellule di ovario di Hamster cinese (CHO), che sono non tumorali ma proliferanti. In particolare sono stati analizzati gli effetti del trattamento con RESV rispetto alla stabilità dei cromosomi, in termini di danno al DNA e di induzione dell'inibizione della segregazione dei cromosomi.
Abbiamo trovato un aumento di cellule poliploidi (POL) e endoreduplicate (ENDO) come conseguenza dell'inibizione della segregazione cromosomica esercitata dal RESV. L'endoreduplicazione consiste in una nuova sintesi di DNA cui non segue una divisione cellulare. Poi attraverso il test dei micronuclei abbiamo trovato un significativo aumento della frequenza di danno cromosomico, come precedentemente dimostrato in cellule di glioma. Il co-trattamento con l'inibitore della TOPO2, EtBr, ha causato una riduzione significativa della frequenza di micronuclei, indicando che sia il RESV che l'EtBr competono per il legame alla TOPO2. Inoltre attraverso

la colorazione anti-cinetocore, abbiamo dimostrato che il RESV causa la produzione di micronuclei positivi per la presenza del centromero, ad indicare che il micronucleo è costituito da un intero cromosoma. Quindi il RESV non solo induce DSBs, ma causa anche la perdita di interi cromosomi. Per analizzare gli effetti della inibizione della segregazione dei cromosomi rispetto alla progressione mitotica, abbiamo analizzato l'eventuale variazione nella frequenza di figure mitotiche dopo il trattamento con RESV. Abbiamo trovato un accumulo di cellule nello stadio di prometa/metafase a scapito dell'ana/telofase. La manifestazione dell'inibizione della segregazione cromosomica è la presenza alla ana/telofase di ponti anafasici di cui abbiamo trovato un significativo aumento dopo il trattamento con la dose più alta di RESV.
Nel complesso i nostri dati confermano il ruolo chemiopreventivo del RESV in cellule tumorali e dimostrano per la prima volta che il RESV è in grado di "avvelenare" la TOPO2, inducendo in questo modo DSBs, attivando meccanismi coinvolti nella segnalazione del danno al DNA e di controllo del ciclo cellulare nelle cellule tumorali. Inoltre esso influenza la stabilità cromosomica a vari livelli in cellule non tumorali proliferanti come conseguenza dell'avvelenamento della TOPO2. Queste evidenze creano un nuovo punto di vista sulle attività del RESV, dando forza all'idea che possa essere utilizzato nella prevenzione del cancro e/o come coadiuvante nelle terapie convenzionali.

1 INTRODUCTION

1.1 The Cancer Chemoprevention

In the early stages of the history of medicine it was already clear a relationship between diet and human diseases and in ancient time several remedies for illnesses were based on the use of natural products.
Over the last century it has been laid the foundations for a scientific explanation of the beneficial effects of natural compounds for human beings, in particular against cancer onset. Many epidemiological investigations showed that a diet rich in fruit and vegetables (400g/day) was associated with a reduced cancer risk [1]. In particular people who eat about five servings of fruit and vegetables a day have half as much risk of developing cancer of the digestive and respiratory tract than those who eat fewer than two servings [2].
The beneficial effects of fruit and vegetables have been attributed to "bioactive" compounds, namely non-nutrient components of food. At present many phytochemicals are known to have key roles in the prevention of chronic diseases, such as cancer, diabetes and hypercholesteremia.
In 1976 Sporn defined chemoprevention as "the use of natural or synthetic compounds to inhibit, suppress or reverse the development and progression of cancer" [3].
In general a phytochemical could act both as a chemotherapeutic and as a chemopreventive agent. Indeed several drugs used in chemotherapy are derived by natural compounds. The differences lie in both the dose utilised and in side effects. A chemotherapeutic agent is applied at pharmacological doses and usually shows several side-effects whereas a chemopreventive one is assimilated through diet, that is at low doses and it doesn't show any toxic effect. The overall concept is that in healthy population a chemopreventive agent, taken through diet, acts by reducing the risk of cancer onset, while in cancer patients it should be applied during the chemotherapeutic cycle in order to reduce side effects and to provide additive or synergistic effects [4].
In the last two decades many projects have been launched in order to discover bioactive compounds able to act as chemopreventive agents. Actually a huge amount of phytochemicals owning a chemopreventive activity is known and they are grouped in several classes such as vitamins, polyphenols, flavonoids, stilbenes, carotenoids, flavones and many others (Fig. 1).

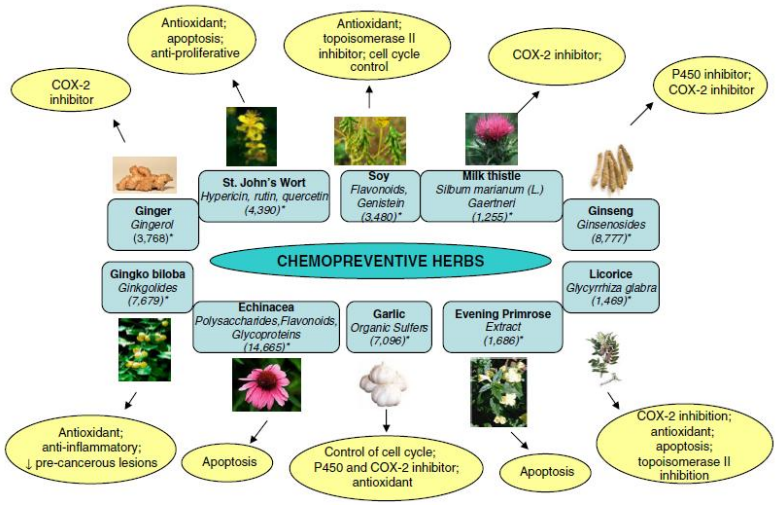

Figure 1. Some of the herbs widely consumed in USA and reported to have chemopreventive efficacy in the literature. The squares indicate the name of the herbs and active phytochemicals present in them. The circles indicate reported mechanism(s) of action for these agents. Numbers in the parentheses (*) represent the estimated US population consuming these herbs. (from Mehta et al. 2010)

Once identified these bioactive compounds, the purpose of the last years was to identify which molecular pathways involved in carcinogenesis they were active. This intense research showed that many phytochemicals can affect one or more of the deregulated pathways of carcinogenesis. Specifically, it has been found they are active in those related to carcinogen metabolism, DNA repair, cell proliferation, apoptosis, cell cycle, angiogenesis and metastasis [5].

Carcinogenesis is a quite complex process that in a simplistic manner can be divided into three major stages. The initiation involves gene mutation, carcinogen metabolism and aberrant DNA repair. In this initial stage, environmental carcinogens induce one or more simple mutations in genes which control the process of carcinogenesis. The stage of promotion is characterized by deregulation of signalling pathways which normally control cell proliferation and apoptosis. Genes that control cell cycle are often mutated in human cancers. Finally, the stage of progression is characterized by genetic alterations within the karyotype of the cells resulting in chromosomal abnormalities. This stage is characterised by invasion, angiogenesis, and metastatic growth (Fig. 2).

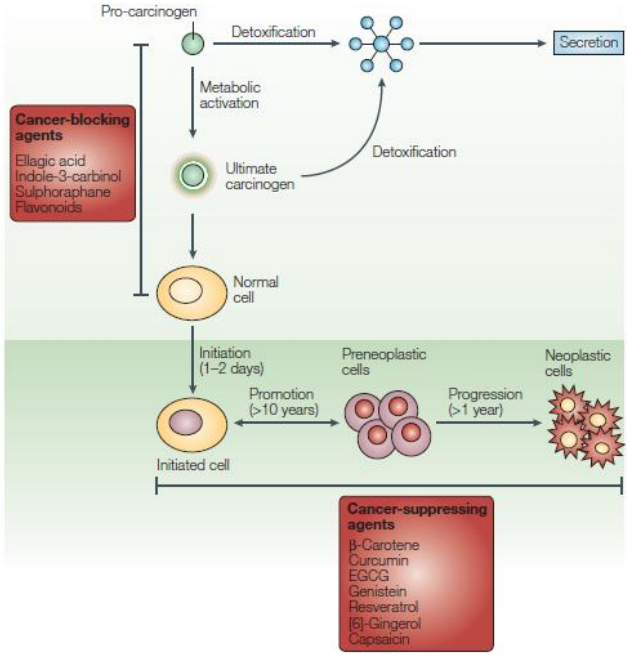

Figure 2. The process of carcinogenesis is defined as initiation, promotion and progression. Progression is shown here to include the growth of malignant tumors, invasion and metastasis. In this diagram for each of the stages, various major actions of phytochemicals involving signaling pathways are summarized. (from Surh 2003)

In the last few years different studies have shown that natural constituents of the regular diet influence the process of carcinogenesis. They can act as tumor-blocking agents, tumor-suppressing agents or both. Tumor-blocking agents either prevent DNA damage formation or promote DNA damage removal, and tumor-suppressing agents slow down the process of initiated cells to become invasive cancer cells [2].
Among the plethora of known chemopreventive agents one of the most studied is the stilbene resveratrol, that has excited a great interest in the research community for its potential beneficial effects on human health.

1.2 Resveratrol in Chemoprevention

Resveratrol (3,5,4'-*trans*-hydroxystilbene) (RESV) is a phytoalexin that protects the plant from injury, ultraviolet (UV) irradiation, and fungal attacks in nature [6]. It was first described in 1940 as a phenolic component

of the medicinal herb hellebore [7] and then in 1963 in the Japanese knotweed *Polygonum cuspidatum*. At the moment it has been detected in at least 100 plants many of which are normal components of human diet. Some examples of main dietary sources of RESV are grape skin (5-10 mg/g), red wine (0.1-1.43 mg/l), cranberry (1.9 mg/g), red currant (1.5 mg/g), raw peanuts (0.15 mg/g). It is also present in the Ayurvedic formula Darakchasawa (0.36 mg/100 ml) and in the Itadori tea (0.97 mg/100 ml) [8]. RESV exists as *cis* and *trans* isomeric forms, with *trans* to *cis* isomerization facilitated by UV exposure. Its stilbene structure is related to the synthetic estrogen diethylstilbestrol. Two phenolic rings are linked by a styrene double bond to generate 3, 4',5- trihydroxystilbene (Fig.3).

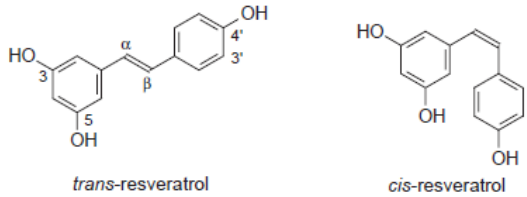

Figure 3. Structural formulas of *trans*-resveratrol and *cis*-resveratrol.

Only in 1992 this molecule polarized the attention of scientific community because it has been identified as the bioactive compound of red wine. This discovery was able to provided an explanation at the so called "French paradox", a term coined in order to explain the correlation between a moderate red wine consumption with a low incidence of cardiovascular diseases despite a high fat diet [9]. Then in 1997 Jang and co-workers demonstrated that RESV acted *in vivo* as a chemopreventive agent in the three stages of carcinogenesis [10].

Since then the number of publications has been growing exponentially and *in vitro*, *ex vivo* and animal model experiments have been providing evidence for bioactivity with clinical potential in cancer chemoprevention and therapy, cardiovascular disease and obesity, hepatic alcoholic or metabolic dysregulation, diabetes, arthritis, osteoporosis and neuroprotection [8].

The chemopreventive property of RESV has been reflected by its ability to block the activation of various carcinogens and/or to stimulate their detoxification, to prevent oxidative damage of target cell DNA, to reduce inflammatory responses and to diminish proliferation of cancer cells. Blockade of angiogenic and metastatic processes of tumor progression, and alleviation of chemotherapy resistance indicate the chemotherapeutic

potential of RESV. The induction of apoptosis in various premalignant or cancerous cells by RESV can contribute to both chemopreventive and chemotherapeutic potential of this compound (Fig. 4) [11].

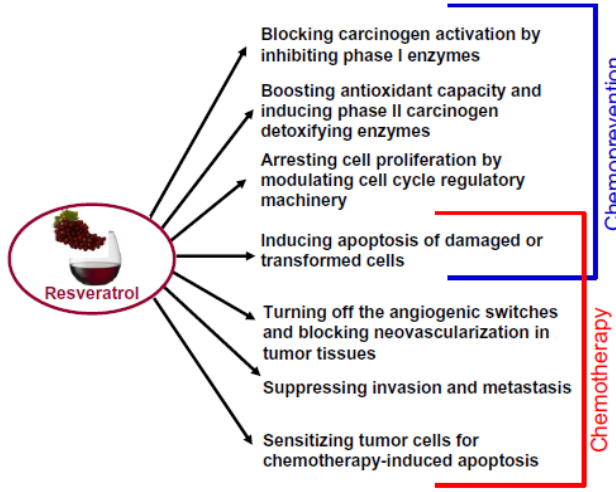

Figure 4. Biochemical mechanisms responsible for chemopreventive and chemotherapeutic potential of resveratrol. (from Kundu & Surh 2008)

The normal cell homeostasis has maintained by a fine tuning of the intracellular signalling network, formed by upstream kinases, that translate extracellular signals into biological responses through the activation of transcription factors. There are *in vitro* and *in vivo* data indicating the capacity of RESV to "turn on" or "switch off" several of these players. It inhibits the phopshporylation of the upstream kinases MAPKs, PKC and Akt and suppresses also the activation of transcription factors NF-κK and AP-1 [11].

Despite a huge amount of *in vitro* and *in vivo* studies, there are few data indicating a potential therapeutic use of RESV in humans. Indeed a big drawback of this molecule is represented by its poor bioavailability. Pharmacokinetic studies have demonstrated a rapid metabolization exerted by phase II enzymes that leads to the production of sulphate and glucoronide forms. The ingestion up to 5 g a day of RESV didn't show any adverse effect in human volunteers but its maximal concentration in plasma reached values ranging between 0.3 and 2.4 μM [12].

A study group established by the 1st International Conference on Resveratrol and Health in 2010 has worked out a series of guidelines for the use of

RESV in clinical trials [13]. A systematic review of literature data showed that there are not sufficient evidences to assert that RESV exerts a preventive effect in human diseases and regard to the toxicity after a nutraceutical intake there are not valid data yet. On the contrary studies in rodent showed that there are sufficient evidences for a chemopreventive role of RESV on the development of cancer skin and there are promising results on the prevention of colon cancer. They concluded that further clinical trials are necessary to elucidate both the chemopreventive role of RESV and the possible side effects in humans.
A big challenge in the cancer fight is to discover molecules able to selectively kill cancer cells with none or few side effects in normal tissues. RESV is a promising chemopreventive agent because it exerts antiproliferative and pro-apoptotic activities at micromolar concentrations in a wide range of cancer cells.

1.3 Resveratrol in Cell Proliferation and Apoptosis

The cell cycle progression is a tightly regulated process based on cyclic events of production and degradation of key enzymes, cyclins (A, B, Ds, E) and cyclin-dependant kinases (Cdk 1, 2, 4, 6). Cyclins are regulator proteins that activate their respective Cdks. The intracellular level of cyclins grows during the interphase reaching a maximum at the onset of mitosis when they undergo degradation [14].
There are many data indicating an antiproliferative effect of RESV in different phases of cell cycle in cancer cells depending on the type considered. The cell cycle block can occur at the G1/S phase through the down regulation of cyclins D1/D2/E, Cdks 2/4/6 and the induction of p21WAF1 and p27KIP1. The arrest in S as well as G2/M phase occurs by inhibition of Cdk7 and p34Cdc2 kinases. RESV is also an upregulator of the oncosuppressor p53 and in addition it causes the cell cycle arrest in cell-lacking p53 [15].
The cell can undergo apoptosis through two ways. The principal is the so called "intrinsic pathway", that involves mitochondria and leads to the release of soluble compounds from the intermembrane space to the cytosol. In most cases, the molecules released activate caspases, leading to the proteolytic cleavage of a series of intracellular proteins, the condensation of nuclear chromatin and the fragmentation of DNA [16]. The intrinsic pathway involves also the tumor suppressor p53, the most commonly mutated gene in human cancer. Normal p53 function leads to cell cycle arrest at the G1 and G2 checkpoints in response to DNA damage. This checkpoint function is executed by the accumulation of p53 followed by the

induction of the gadd45, waf1 and mdm2 genes. If DNA repair is not successful, p53 initiates apoptosis, thus preventing the propagation of genetic defects to successive cell generations [17].
The "extrinsic pathway" is triggered by the ligation of death receptor Fas (also known as APO-1 or CD95), to its natural ligand, Fas ligand (FasL, APO-1L, CD95L). Activation of this receptor results in a recruitment of caspase zymogenes into oligomeric complexes and triggers their proteolytic activation [18].
Regarding the first way, RESV affects the respiratory chain producing the release of Reactive Oxygen Species (ROS) and modulates the mitochondria membrane permeability causing a rapid depolarization. This effect is mediated through a downregulation of the expression of anti-apoptotic Bcl-2 proteins [19] and an upregulation of pro-apoptotic Bcl-2 proteins (Bax, Bak, Bad, Bid). RESV is also able to induce apoptosis in a p53 dependent or independent manner, depending on the cell type studied [20].
RESV leads to the activation of the "extrinsic pathway" through a redistribution of receptor molecules Fas in the plasma membrane. This action causes the production of lipid rafts that play an important role in clustering surface receptors, signalling enzymes and adaptor molecules into membrane complexes at specific sites. RESV-induced redistribution of Fas in the rafts could contribute to the formation of the death-inducing signalling complex (DISC) observed in colon cancer cells treated with the polyphenol. It has been also found that in some cell types RESV induces the sphingomyelin/ceramide apoptotic pathway, which in turn plays a crucial role in lipid rafts formation [20,21].
Finally various reports have shown that RESV is able to act on NF-κB pathways, that is an inhibitor of apoptosis, at multiple levels such as down-regulation of NF-κB protein expression, modulation of phosphorylation and transcriptional activity [22].
There are also data indicating that RESV kills cancer cells not only by apoptosis, but also through other mechanisms such as phagocytosis and intracellular killing, senescence and mitotic catastrophe [20].

1.4 Resveratrol and DNA

RESV is involved in several aspects of DNA metabolism such as replication, recombination, relaxation, repair and telomere maintenance. It affects directly several enzymes of DNA metabolism, such as DNA polymerases α and δ, ribonucleotide reductase and telomerase (Fig. 5) [23].
RESV is able to maintain DNA integrity because is a scavenger of reactive oxygen species (ROS). At the same time it owns an intrinsic antioxidant

capacity and acts as activator of detoxifying enzymes, such as superoxide dismutase, catalase and glutathione peroxidase. These data have been obtained *in vitro*, whereas it is a matter of debate if RESV possesses a radical scavenger property also *in vivo*. Moreover in certain conditions, such as other plant polyphenols, it showed a pro-oxidant activity [7].
It has been shown that RESV interacts directly with the DNA molecule. It establishes several hydrogen bonds without any alteration of double helix structure. Indeed it seems to stabilize the molecule and it could represent the molecular basis of the protection against genotoxic injuries [24]. Moreover RESV reverts the intercalation exerted by ethidium bromide without acting itself as an intercalating agent [25]. The Ames test, which measures the reversion of mutations in bacteria, didn't show any mutagenic effect of RESV [26].

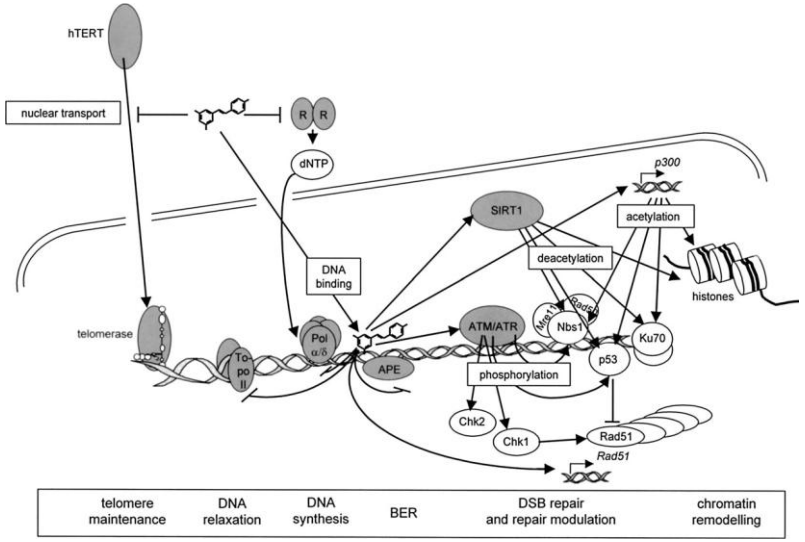

Figure 5. Nuclear activities of resveratrol with relevance for DNA repair. Gray color marks the immediate targets of resveratrol. (from Gatz & Wiesmuller 2008)

1.4.1 RESV and DNA damage in non-cancer cells

With respect to the genotoxic activity in non-cancer cells, interestingly, increasing concentrations of RESV (10–100 μM) in the presence of copper ions were showed to induce DNA damage in human lymphocytes. Moreover it has been demonstrated that the presence of hydroxyl groups is essential

for DNA cleavage [27]. Further, the RESV-induced DNA degradation in lymphocytes is inhibited by scavengers of ROS and neocuproine, a Cu(I)-specific sequestering agent [28]. Copper is an important metal ion present in chromatin and is closely associated with DNA bases, particularly guanine [29]. It is also one of the most redox active of all the various metal ions present in the cells. Evidences have shown that polyphenols including RESV do not only bind copper ions but also catalyze their redox cycling [30]. A mechanism was proposed which involves the formation of a ternary complex of DNA–polyphenol–Cu(II) [31]. A redox reaction of this compound and Cu(II) in the ternary complex may occur, leading to the reduction of Cu(II) to Cu(I), whose re-oxidation generates a variety of ROS. These findings demonstrated that the RESV–Cu(II) system for DNA breakage is physiologically feasible and could be of biological significance. In this context Schilder and co-workers have shown that a chronic treatment with RESV induces an increase of ROS in human endothelial cells and this increase is linked to S phase accumulation and finally to cell senescence [32].

As far as the ability of RESV in inducing DNA breaks it is still debated but not much data are present in literature about the induction of chromosomal damage in normal cells. In particular Matsuoka and co-workers showed that RESV is able to induce sister chromatid exchange (SCEs) and micronuclei (MN) in Chinese hamster lung cells at micromolar concentrations [26]. In my host lab a slight increase in chromosome aberrations after 200 μM RESV treatment in Chinese hamster ovary cells has been shown [33].

1.4.2 RESV and DNA damage in cancer cells

DNA damage is a key factor both in the evolution and treatment of cancer. Cellular responses to DNA damage are coordinated primarily by two distinct kinase signaling cascades, the ATM-Chk2 and ATR-Chk1 pathways, which are activated by DNA double and single-strand breaks respectively. In response to DSBs, ATM is required both for ATR-Chk1 activation and to initiate DNA repair via homologous recombination repair (HRR) by promoting formation of single-stranded DNA at damage sites through nucleolytic resection. The ATR-Chk1 pathway is the principal direct effector of the DNA damage and replication checkpoints and, as such, is essential for the survival of many, although not all, cell types [34]. The ATM-mediated posphorylation of H2AX (γ-H2AX) is an hallmark of the cellular response to DNA DSBs. This step is followed by the accumulation and local concentration of a plethora of DNA damage signalling and repair proteins in the vicinity of the lesion and culminating in the generation of

distinct nuclear compartments, so-called Ionizing Radiation-Induced Foci [35].

The first demonstration of a DNA cleavage exerted by RESV emerged in 1998. In a plasmid-based DNA cleavage assay, RESV mediated the relaxation of pBR322 at micromolar concentrations in the presence of Cu(II). It was showed that RESV was capable of binding to DNA, and that the Cu(II)-dependent DNA damage is more likely caused by a copper-peroxide complex rather than by freely diffusible oxygen species [36]. On the other hand a chronic administration of non apoptotic doses of RESV induces senescence in colon carcinoma cells through the increase of Radical Oxygen Species (ROS) production by mitochondria [37]. So that even if RESV has been mostly considered an antioxidant, it could act as a radical scavenger in some conditions and during the initial phases of exposure, while its pro-oxidant activity would start according to prolonged exposure time and higher concentrations.

Regarding the genotoxic effect of RESV in cancer cells, there are data that seem to support the hypothesis that DNA strand breaks could be a starting point in the apoptosis induction.

The treatment with a high dose of RESV induces DNA damage, as measured through Comet assay, both in human hepatic cancer cells [38] and in C6 rat glioma cells [39]. In the same cells the effect of RESV on chromosome damage has been measured by micronucleus assay. A dose of 250 μM RESV induced an increase (30%) in micronucleated cells after 24 h of treatment [40].

However, the vast majority of data on RESV ability in inducing DNA damage deal with the phosphorylation of the histone H2AX (γ-H2AX). In peripheral blood and bone marrow mononuclear cells isolated from chronic lymphocytic leukaemia patients, the induction of γ-H2AX and the activation of ATM caused by RESV has been measured. It has been shown that a 40 μM dose of RESV caused an increase in γ-H2AX expression concurrent with increased expression of activated ATM in most but not in all patients. Furthermore in the γ-H2AX-positive patients the increase in the apoptotic response after RESV treatment was significantly higher [41]. Also in human ovarian cancer cells RESV activates ATM/ATR kinases following DNA damage as detected by serine 139 phosphorylation of H2AX. Moreover RESV treatment resulted in an increase in Chk1 phosphorylation (Ser296) as well as in total protein level. Similarly, RESV strongly induced the phosphorylation of Chk2 at Thr68, Thr387 and Ser19 sites together with a moderate increase in total Chk2 protein level [42].

Furthermore, RESV can activate different cell cycle checkpoint mechanisms in response to DNA damage on the basis of the cell line analysed. Human

osteosarcoma and lung adenocarcinoma cells showed an upregulation of γ-H2AX at the dose of 50 μM of RESV. Differential expression of BRCA1, cyclin B1, pRb and p21 in U-2OS and A549 cells indicates that RESV can engage various molecular mechanisms to arrest cell cycle progression [43]. More recently Tyagi and co-workers have shown that RESV is able to induce DNA damage as measured through γ-H2AX expression and consequently apoptotic death in head and neck squamous cell carcinoma cells [44]. In human gastric adenocarcinoma cells a treatment with 50-200 μM of RESV caused the phosphorylation of the histone H2AX followed by apoptosis induction [45]. At the same manner in human lung adenocarcinoma cells 50 μM RESV was able to induce the phopsphotylation of ATM on Ser-1981 and a concomitant expression of γ-H2AX [46].
Recently it has been shown that RESV and other polyphenols such as genistein and baicalein, increased the level of ATAD5 protein that is a biomarker of DNA damage. This genotoxic effect was accompanied by apoptosis induction in proliferating cells but unlike conventional genotoxic chemotherapeutic agents, such as cisplatin, that produce mutagenesis at the chromosomal and nucleotide levels, RESV did not increase mutagenesis in the *SupF* plasmid mutagenesis assay [47].
On the whole these data seem to support the hypothesis that the anti-tumoral activity of RESV is in part due to its capacity to induce DNA damage in cancer cells, so activating the checkpoints responsible for cell cycle arrest and, in some cases, apoptosis. A putative mechanism by which RESV induces DNA DSBs could be the interaction with topoisomerase IIα, that is an enzyme mainly expressed in proliferating cells and involved in several aspects of DNA metabolism.

1.5 DNA Topoisomerases

DNA topoisomerases are conserved nuclear enzymes that are involved in the maintaining of DNA topology. In higher eukaryotes there are two classes of topoisomerases: type I enzymes introduce single strand breaks in DNA and type II ones introduce DSBs. Since a single unrepaired DSB has potentially lethal consequences, type II topoisomerases might be viewed as a particularly dangerous way of dealing with the topological problems of DNA. Mammalian cells express two isoforms (α and β) of topoisomerase II. The expression of Topoisomerase IIα (TOPO2) is cell cycle regulated, and this enzyme is essential for the viability of all dividing cells. Many non-dividing cells lack detectable TOPO2 [48].
Reactions catalyzed by eukaryotic TOPO2 include decatenation of linked intact double stranded DNA and relaxation of supercoiled DNA. The

enzyme introduces a double strand break in one DNA strand, termed the G or "gate segment", and will pass a second strand termed the T segment through the break. ATP binding causes the enzyme to form a closed clamp. The closed clamp may also capture another strand (the T strand) that will pass through the break made in the G strand. ATP hydrolysis occurs at two steps in the reaction cycle. The first ATP hydrolyzed may assist in strand passage. The second hydrolysis step (along with release of ADP and Pi) allows the clamp to re-open, and allows release of the G segment (for a distributive reaction). Alternately, the enzyme may initiate another catalytic cycle without dissociating from the G strand (Fig. 6).

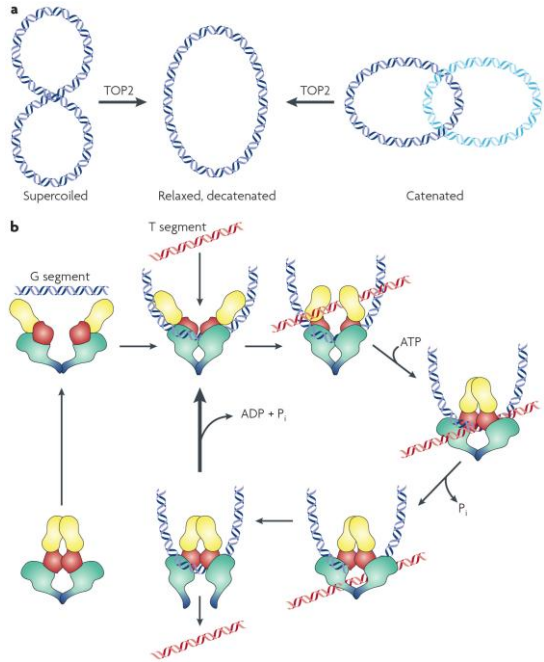

Figure 6. a. Reactions catalyzed by eukaryotic TOPO2 include decatenation of linked intact double stranded DNA and relaxation of supercoiled DNA. The reaction formally requires introduction of a double strand break, strand passage, and break resealing. **b**. Mechanism of strand passage by TOPO2. (from Nitiss 2009)

One of the central roles of TOPO2 is to solve the topological problems associated with replication. Semi-conservative replication involves the

unwinding of duplex DNA and copying of each strand. In the absence of TOPO2 activity the unwinding of the parental duplex leads to the accumulation of positively supercoiled DNA in front of the replication fork. Studies indicated also that TOPO2 plays a key role in chromosome structure and chromosome condensation and it is involved in decatenation of sister chromatids during mitosis. Since TOPO2 carries out a reaction that is essential for chromosome separation at mitosis, a plausible hypothesis is that cells can monitor the successful completion of TOPO2 decatenation, and arrest cell cycle progression if decatenation (or chromosome condensation) is incomplete [48].

TOPO2 has held the interest of researchers studying cancer owing to the discovery that it is targeted by active anticancer drugs. Drugs targeting TOPO2 are divided into two broad classes. The first class, which includes most of the clinically active agents, leads to increases in the levels of TOPO2–DNA covalent complexes, named "cleavage complex". Because these agents generate lesions that include DNA strand breaks and protein covalently bound to DNA, they have been termed TOPO2 poisons. A second class of compounds inhibits TOPO2 catalytic activity but does not generate increases in the levels of TOPO2 covalent complexes. Agents in this second class are thought to kill cells through the elimination of the essential enzymatic activity of TOPO2 and are therefore termed TOPO2 catalytic inhibitors.

The cleavage complexes are tightly regulated in the cells; if their level falls too low, cells are unable to undergo chromosome segregation and ultimately die of mitotic failure. On the contrary a higher level of cleavage complexes leads to an accumulation of DNA breaks that can evolve into chromosome translocations and other DNA aberrations. If the accumulation of breaks is overwhelming, they trigger apoptotic pathways and kill the cell [49]. If these DNA strand breaks do not result in cell death, chromosomal translocations may be present in surviving cellular populations [50]. For these reasons, in particular conditions, TOPO2 can change into a genotoxic enzyme.

Polyphenolic compounds, in particular bioflavonoids such as genistein and quercetin, have been shown to enhance DNA cleavage mediated by human TOPO2 isoform α and β [51]. This activity is exerted by increasing the levels of DNA cleavage complexes generated by TOPO2 through a direct interaction of genistein with an ATP binding motif of the enzyme. It has been demonstrated that polyphenolic fractions from grape cell culture are potent inhibitors of human TOPO2 catalytic activity [52].

In two studies RESV was characterized as a catalytic inhibitor of TOPO2 [53,54]. meanwhile another report postulated TOPO I poisoning activity for

RESV [55]. However, only a limited number of experiments were performed in this study.

1.6 REFERENCES

1. Gullett, N.P., Ruhul Amin, A.R., Bayraktar, S., Pezzuto, J.M., Shin, D.M., Khuri, F.R., Aggarwal, B.B., Surh, Y.J. and Kucuk, O. (2010) Cancer prevention with natural compounds. *Semin Oncol*, 37, 258-81.
2. Surh, Y.J. (2003) Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer*, 3, 768-80.
3. Sporn, M.B., Dunlop, N.M., Newton, D.L. and Smith, J.M. (1976) Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Fed Proc*, 35, 1332-8.
4. Mehta, R.G., Murillo, G., Naithani, R. and Peng, X. (2010) Cancer chemoprevention by natural products: how far have we come? *Pharm Res*, 27, 950-61.
5. Kwon, K.H., Barve, A., Yu, S., Huang, M.T. and Kong, A.N. (2007) Cancer chemoprevention by phytochemicals: potential molecular targets, biomarkers and animal models. *Acta Pharmacol Sin*, 28, 1409-21.
6. Shakibaei, M., Harikumar, K.B. and Aggarwal, B.B. (2009) Resveratrol addiction: to die or not to die. *Mol Nutr Food Res*, 53, 115-28.
7. Baur, J.A. and Sinclair, D.A. (2006) Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov*, 5, 493-506.
8. Chachay, V.S., Kirkpatrick, C.M., Hickman, I.J., Ferguson, M., Prins, J.B. and Martin, J.H. (2011) Resveratrol--pills to replace a healthy diet? *Br J Clin Pharmacol*, 72, 27-38.
9. Renaud, S. and de Lorgeril, M. (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet*, 339, 1523-6.
10. Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C. and Pezzuto, J.M. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, 275, 218-20.
11. Kundu, J.K. and Surh, Y.J. (2008) Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic. *Cancer Lett*, 269, 243-61.

12. Boocock, D.J., Faust, G.E., Patel, K.R., Schinas, A.M., Brown, V.A., Ducharme, M.P., Booth, T.D., Crowell, J.A., Perloff, M., Gescher, A.J., Steward, W.P. and Brenner, D.E. (2007) Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. *Cancer Epidemiol Biomarkers Prev*, 16, 1246-52.
13. Vang, O., Ahmad, N., Baile, C.A., Baur, J.A., Brown, K., Csiszar, A., Das, D.K., Delmas, D., Gottfried, C., Lin, H.Y., Ma, Q.Y., Mukhopadhyay, P., Nalini, N., Pezzuto, J.M., Richard, T., Shukla, Y., Surh, Y.J., Szekeres, T., Szkudelski, T., Walle, T. and Wu, J.M. (2011) What is new for an old molecule? Systematic review and recommendations on the use of resveratrol. *PLoS One*, 6, e19881.
14. Murray, A.W. (2004) Recycling the cell cycle: cyclins revisited. *Cell*, 116, 221-34.
15. Athar, M., Back, J.H., Kopelovich, L., Bickers, D.R. and Kim, A.L. (2009) Multiple molecular targets of resveratrol: Anti-carcinogenic mechanisms. *Arch Biochem Biophys*, 486, 95-102.
16. Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, 281, 1309-12.
17. Greenblatt, M.S., Bennett, W.P., Hollstein, M. and Harris, C.C. (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res*, 54, 4855-78.
18. Ashkenazi, A. and Dixit, V.M. (1998) Death receptors: signaling and modulation. *Science*, 281, 1305-8.
19. Shankar, S., Singh, G. and Srivastava, R.K. (2007) Chemoprevention by resveratrol: molecular mechanisms and therapeutic potential. *Front Biosci*, 12, 4839-54.
20. Delmas, D., Solary, E. and Latruffe, N. (2011) Resveratrol, a phytochemical inducer of multiple cell death pathways: apoptosis, autophagy and mitotic catastrophe. *Curr Med Chem*, 18, 1100-21.
21. Delmas, D., Rebe, C., Lacour, S., Filomenko, R., Athias, A., Gambert, P., Cherkaoui-Malki, M., Jannin, B., Dubrez-Daloz, L., Latruffe, N. and Solary, E. (2003) Resveratrol-induced apoptosis is associated with Fas redistribution in the rafts. *J Biol Chem*, 278, 41482-90.
22. Sun, C., Hu, Y., Liu, X., Wu, T., Wang, Y., He, W. and Wei, W. (2006) Resveratrol downregulates the constitutional activation of nuclear factor-kappaB. *Cancer Genet Cytogenet*, 165, 9-19.
23. Gatz, S.A. and Wiesmuller, L. (2008) Take a break--resveratrol in action on DNA. *Carcinogenesis*, 29, 321-32.

24. Usha, S., Johnson, I.M. and Malathi, R. (2005) Interaction of resveratrol and genistein with nucleic acids. *J Biochem Mol Biol*, 38, 198-205.
25. Usha, S., Johnson, I.M. and Malathi, R. (2006) Modulation of DNA intercalation by resveratrol and genistein. *Mol Cell Biochem*, 284, 57-64.
26. Matsuoka, A., Furuta, A., Ozaki, M., Fukuhara, K. and Miyata, N. (2001) Resveratrol, a naturally occurring polyphenol, induces sister chromatid exchanges in a Chinese hamster lung (CHL) cell line. *Mutat Res*, 494, 107-13.
27. Azmi, A.S., Bhat, S.H. and Hadi, S.M. (2005) Resveratrol-Cu(II) induced DNA breakage in human peripheral lymphocytes: implications for anticancer properties. *FEBS Lett*, 579, 3131-5.
28. Hadi, S.M., Ullah, M.F., Azmi, A.S., Ahmad, A., Shamim, U., Zubair, H. and Khan, H.Y. (2010) Resveratrol mobilizes endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: a putative mechanism for chemoprevention of cancer. *Pharm Res*, 27, 979-88.
29. Kagawa, T.F., Geierstanger, B.H., Wang, A.H. and Ho, P.S. (1991) Covalent modification of guanine bases in double-stranded DNA. The 1.2-A Z-DNA structure of d(CGCGCG) in the presence of CuCl2. *J Biol Chem*, 266, 20175-84.
30. Hanif, S., Shamim, U., Ullah, M.F., Azmi, A.S., Bhat, S.H. and Hadi, S.M. (2008) The anthocyanidin delphinidin mobilizes endogenous copper ions from human lymphocytes leading to oxidative degradation of cellular DNA. *Toxicology*, 249, 19-25.
31. Rahman, A., Shahabuddin, Hadi, S.M., Parish, J.H. and Ainley, K. (1989) Strand scission in DNA induced by quercetin and Cu(II): role of Cu(I) and oxygen free radicals. *Carcinogenesis*, 10, 1833-9.
32. Schilder, Y.D., Heiss, E.H., Schachner, D., Ziegler, J., Reznicek, G., Sorescu, D. and Dirsch, V.M. (2009) NADPH oxidases 1 and 4 mediate cellular senescence induced by resveratrol in human endothelial cells. *Free Radic Biol Med*, 46, 1598-606.
33. De Salvia, R., Festa, F., Ricordy, R., Perticone, P. and Cozzi, R. (2002) Resveratrol affects in a different way primary versus fixed DNA damage induced by H(2)O(2) in mammalian cells in vitro. *Toxicol Lett*, 135, 1-9.
34. Smith, J., Tho, L.M., Xu, N. and Gillespie, D.A. (2010) The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res*, 108, 73-112.

35. Bekker-Jensen, S. and Mailand, N. (2010) Assembly and function of DNA double-strand break repair foci in mammalian cells. *DNA Repair (Amst)*, 9, 1219-28.
36. Fukuhara, K. and Miyata, N. (1998) Resveratrol as a new type of DNA-cleaving agent. *Bioorg Med Chem Lett*, 8, 3187-92.
37. Heiss, E.H., Schilder, Y.D. and Dirsch, V.M. (2007) Chronic treatment with resveratrol induces redox stress- and ataxia telangiectasia-mutated (ATM)-dependent senescence in p53-positive cancer cells. *J Biol Chem*, 282, 26759-66.
38. Choi, H.Y., Chong, S.A. and Nam, M.J. (2009) Resveratrol induces apoptosis in human SK-HEP-1 hepatic cancer cells. *Cancer Genomics Proteomics*, 6, 263-8.
39. Michels, G., Watjen, W., Weber, N., Niering, P., Chovolou, Y., Kampkotter, A., Proksch, P. and Kahl, R. (2006) Resveratrol induces apoptotic cell death in rat H4IIE hepatoma cells but necrosis in C6 glioma cells. *Toxicology*, 225, 173-82.
40. Quincozes-Santos, A., Andreazza, A.C., Goncalves, C.A. and Gottfried, C. (2010) Actions of redox-active compound resveratrol under hydrogen peroxide insult in C6 astroglial cells. *Toxicol In Vitro*, 24, 916-20.
41. Podhorecka, M., Halicka, D., Klimek, P., Kowal, M., Chocholska, S. and Dmoszynska, A. (2011) Resveratrol increases rate of apoptosis caused by purine analogues in malignant lymphocytes of chronic lymphocytic leukemia. *Ann Hematol*, 90, 173-83.
42. Tyagi, A., Singh, R.P., Agarwal, C., Siriwardana, S., Sclafani, R.A. and Agarwal, R. (2005) Resveratrol causes Cdc2-tyr15 phosphorylation via ATM/ATR-Chk1/2-Cdc25C pathway as a central mechanism for S phase arrest in human ovarian carcinoma Ovcar-3 cells. *Carcinogenesis*, 26, 1978-87.
43. Rusin, M., Zajkowicz, A. and Butkiewicz, D. (2009) Resveratrol induces senescence-like growth inhibition of U-2 OS cells associated with the instability of telomeric DNA and upregulation of BRCA1. *Mech Ageing Dev*, 130, 528-37.
44. Tyagi, A., Gu, M., Takahata, T., Frederick, B., Agarwal, C., Siriwardana, S., Agarwal, R. and Sclafani, R.A. (2011) Resveratrol selectively induces DNA Damage, independent of Smad4 expression, in its efficacy against human head and neck squamous cell carcinoma. *Clin Cancer Res*, 17, 5402-11.
45. Wang, Z., Li, W., Meng, X. and Jia, B. (2011) Resveratrol induces gastric cancer cell apoptosis via ROS, but independent of sirtuin1. *Clin Exp Pharmacol Physiol*.

46. Zajkowicz, A. and Rusin, M. (2011) The activation of the p53 pathway by the AMP mimetic AICAR is reduced by inhibitors of the ATM or mTOR kinases. *Mech Ageing Dev*, 132, 543-51.
47. Fox, J.T., Sakamuru, S., Huang, R., Teneva, N., Simmons, S.O., Xia, M., Tice, R.R., Austin, C.P. and Myung, K. (2012) High-throughput genotoxicity assay identifies antioxidants as inducers of DNA. *Proc Natl Acad Sci U S A*, 109, 5423-8.
48. Nitiss, J.L. (2009) DNA topoisomerase II and its growing repertoire of biological functions. *Nat Rev Cancer*, 9, 327-37.
49. Baguley, B.C. and Ferguson, L.R. (1998) Mutagenic properties of topoisomerase-targeted drugs. *Biochim Biophys Acta*, 1400, 213-22.
50. Felix, C.A. (1998) Secondary leukemias induced by topoisomerase-targeted drugs. *Biochim Biophys Acta*, 1400, 233-55.
51. Bandele, O.J. and Osheroff, N. (2007) Bioflavonoids as poisons of human topoisomerase II alpha and II beta. *Biochemistry*, 46, 6097-108.
52. Jo, J.Y., Gonzalez de Mejia, E. and Lila, M.A. (2005) Effects of grape cell culture extracts on human topoisomerase II catalytic. *J Agric Food Chem*, 53, 2489-98.
53. Cho, K.H., Pezzuto, J.M., Bolton, J.L., Steele, V.E., Kelloff, G.J., Lee, S.K. and Constantinou, A. (2000) Selection of cancer chemopreventive agents based on inhibition of topoisomerase. *Eur J Cancer*, 36, 2146-56.
54. Jo, J.Y., Gonzalez de Mejia, E. and Lila, M.A. (2005) Effects of grape cell culture extracts on human topoisomerase II catalytic activity and characterization of active fractions. *J Agric Food Chem*, 53, 2489-98.
55. Webb, M.R. and Ebeler, S.E. (2004) Comparative analysis of topoisomerase IB inhibition and DNA intercalation by. *Biochem J*, 384, 527-41.
56. Leone, S., Fiore, M., Lauro, M.G., Pino, S., Cornetta, T. and Cozzi, R. (2008) Resveratrol and X rays affect gap junction intercellular communications in human glioblastoma cells. *Mol Carcinog*, 47, 587-98.

2 AIM OF THE RESEARCH

DNA damage is one of the targets of both chemoprevention and chemotherapy and a desirable outcome is the induction of apoptosis and/or the arrest of cell proliferation. Data demonstrating any genotoxic effects of RESV in cancer cells are poor and controversial, probably due to the use of different cell types and to the heterogeneity of the schedules of treatment. In the host lab it has been previously demonstrated that RESV is able to re-establish Gap Junction Intercellular Communication (GJIC) accompanied by a delay in the S-phase of cell cycle in the U87 glioblastoma mulitforme cell line [56]. This aspect is very interesting in relation to the possibility that RESV could enhance the spreading of damage (DNA damage), cell death or cell cycle control signals through GJIC, so increasing the effect of conventional antitumoral therapy.

On the basis of these previous observations, my PhD project concerned the study of chemopreventive/chemotherapeutic activity of RESV in cancer and normal cells, in terms of effects on cell cycle progression and DNA damage induction.
During the first year we analysed the anti-proliferative effects exerted by RESV in glioblastoma cells, associated with the induction of DNA damage, measured through the H2AX phosphorylation.

In the second year we studied the possible molecular interaction between DNA/RESV/TOPO2, through a Docking Simulation. Furthermore we analysed the consequences of this interaction at the level of cellular DNA integrity. The hypothesis was that this interaction could stabilize the cleavage complexes formed by TOPO2 during its activity on DNA.

Finally in the third year we performed a mechanistic study in Chinese Hamster Ovary (CHO) cells, in order to demonstrate whether also in proliferating non-cancer cells, RESV can affect various aspects of DNA metabolism explicable with the interaction with TOPO2 activity. In particular we found that it induces micronuclei but this effect is attenuated by the co-presence of another TOPO2 inhibitor, supporting the idea that DNA damage induction is caused by TOPO2 poisoning. Moreover it affects proper chromosome segregation at mitosis causing chromosome los, inhibiting decatenation and causing a delay in cell proliferation.

3 PUBLICATIONS

3.1 Leone, S., Cornetta, T., Basso, E. and Cozzi, R. (2010) Resveratrol induces DNA double-strand breaks through human topoisomerase II. Cancer Lett, 295, 167-72.

Cancer Letters 295 (2010) 167–172

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Resveratrol induces DNA double-strand breaks through human topoisomerase II interaction

Stefano Leone, Tommaso Cornetta [1], Emiliano Basso [1], Renata Cozzi *

Dipartimento di Biologia – Università degli Studi Roma Tre, Roma, Italy

ARTICLE INFO

Article history:
Received 15 December 2009
Received in revised form 22 February 2010
Accepted 25 February 2010

Keywords:
γH2AX
Cell cycle
Topoisomerase II
Double-strand breaks
Cytofluorimetric assay

ABSTRACT

Resveratrol, a stilbene found in grapes and wine, is one of the most interesting natural compound due to its role exerted in cancer prevention and therapy. In particular, resveratrol is able to delay cell cycle progression and to induce apoptotic death in several cell lines. Here we report that resveratrol treatment of human glioblastoma cells induces a delay in cell cycle progression during S phase associated with an increase in histone H2AX phosphorylation. Furthermore, with an *in vitro* assay of topoisomerase IIα catalytic activity we show that resveratrol is able to inhibit the ability of recombinant human TOPO IIα to decatenate kDNA, so that it could be considered a TOPO II poison.

1. Introduction

Resveratrol (3,4′,5-trihydroxystilbene) is a well known polyphenol synthesized by a wide variety of plant species in response to injury, UV irradiation and fungal attack. Since Jang et al. [1] showed the antitumoral potential of resveratrol (RSV) *in vivo*, many studies have revealed a variety of resveratrol intracellular targets whose modulation gives rise to overlapping responses that lead to growth arrest and death. In particular it was found to arrest proliferation in many cancer cell models mostly in an irreversible way, leading to apoptosis [2]. Recently we showed that RSV in combined treatment with X-rays both induces a dose-dependent inhibition in cell cycle progression, particularly due to a delay during the S phase, and increases intercellular communication in human glioblastoma cells [3].

In any case, the efficacy of RSV is still debated because of the multiplicity of affected targets and contradictory effects related to dose and time of treatment and to cellular phenotype [4,5]. One of the most interesting issues is the involvement of RSV in the maintenance of genomic stability through physical and chemical interactions with DNA, but also in influencing the redox state of cells. The DNA cleavage activity of RSV was enhanced in the presence of high levels of copper ions [6], but we showed that it does not appear to be able "per se" to induce primary DNA damage in mammalian cells [7]. On the other hand, RSV could also affect some aspects of DNA metabolism such as DNA repair, recombination and chromatin structure maintenance [8,9] thereby indirectly modulating the integrity of genomic DNA.

Here we report that RSV treatment in human glioblastoma cells causes a dose-dependent cell cycle delay with a concomitant increase of histone H2AX phosphorylation. Being that this expression is linked to the increase of DNA double-strand breaks [10], we assayed the ability of RSV to interact with human topoisomerase II.

Abbreviations: RSV, resveratrol; TOPO II, topoisomerase II; DSBs, double-strand breaks.

* Corresponding author. Address: Dipartimento di Biologia, Università degli Studi, Roma Tre, Viale Guglielmo Marconi 446, 00146 Rome, Italy. Tel.: +39 06 57336330/57336333; fax: +39 06 57336321.
E-mail address: cozzi@uniroma3.it (R. Cozzi).

[1] These authors contributed equally to the study.

0304-3835/$ - see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2010.02.022

2. Materials and methods

2.1. Cell cultures and treatments

The human glioblastoma cell line U87-MG was purchased from the American Type Culture Collection (ATCC, USA) and was grown in DMEM cell culture medium with 10% FBS, penicillin/streptomycin and 2 mM L-glutamine, at 37 °C and 5% CO_2. Cells, at exponential phase of growth, were always treated for 48 h with 20, 40 or 80 μM of resveratrol (Sigma) in immunofluorescence and flow cytometry experiments.

2.2. Immunofluorescence of H2AX nuclear foci

U87-MG were grown on μ-Slide 8 well (Ibidi) and treated with resveratrol. Immediately after treatment, cells were washed with PBS, fixed in 4% paraformaldehyde for 10′ at 37 °C and then permeabilized with 90% ice-cold methanol. Cells were incubated for 3 h at room temperature with an Alexa Fluor® 488 anti-H2AX-phosphorylated at Ser139 antibody (Biolegend), 1:10 in PBS/1% BSA incubation buffer. Then, nuclear γH2AX foci fluorescence was analysed at confocal microscopy (TCS STED Leica).

2.3. Flow cytometry for cell cycle and H2AX expression analysis

Detection of nuclear γH2AX and DNA cell content were carried out by using respectively an Alexa Fluor® 488 anti-H2AX-phosphorylated at Ser139 antibody (Biolegend) and propidium iodide staining solution (Sigma). Briefly, U87-MG cells in exponential growth phase were left untreated or treated for 48 h with 20, 40 or 80 μM of resveratrol. After treatment cells were detached with Trypsin/EDTA solution (Sigma), washed with PBS, fixed in 4% paraformaldehyde for 15′ on ice and permeabilized with 70% ice-cold methanol. The samples were resuspended in PBS/0.2% TritonX-100/1% BSA and incubates for 30 min at room temperature. Then cells were double stained with an anti-γH2AX Alexa Fluor® 488-conjugated antibody, 1:10 dilution in PBS/1% BSA incubation buffer for 3 h in the dark and counterstained for cell cycle analysis with propidium iodide (50 μg/ml)/RNaseA (0.15 mg/ml) solution. Finally cells were acquired using a Galaxy flow cytometer (Dakocytomation) and analysed by Flowjo software (Tree star).

2.4. Topoisomerase II decatenation assay

Topoisomerase IIα activity was determined by assessing the ability of purified topoisomerase IIα to decatenate kinetoplast kDNA in the presence and absence of resveratrol. Decatenation assay was performed with 150 ng kinetoplast DNA (kDNA) (TopoGEN Inc.) in a 20 μl reaction containing 50 mM Tris–HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM ATP with or without 20, 40, 80 and 120 μM of resveratrol, 30 μg/ml bovine serum albumin and 4 U of human purified Topo IIα (TopoGEN Inc.). Reactions were incubated at 37 °C for 1 h and stopped by adding 5 μl 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 decatenation was determined by the loss of migration of high molecular kDNA catenated which remained in the well. Catenated, decatenated and linear kDNA markers (TopoGEN Inc.) were loaded as a reference.

3. Results

To perform our experimental design, we selected three doses of RSV (20, 40 and 80 μM) on the basis of our previous study [3], where we showed that the cell survival, as measured by MTT assay, remained high (>90%) in U87 cells treated with RSV doses up to 80 μM; decreased cell survival was observed with increasing concentrations, starting from 160 μM.

Our first observation at confocal microscopy was the detection of numerous cells presenting γH2AX foci when comparing U87-MG cells treated with RSV to control untreated ones (Fig. 1). The foci appear particularly abundant after the RSV highest dose (80 μM) treatment (Fig. 1D).

Next we analysed the effect of RSV treatment on cell cycle progression in concomitance of γH2AX expression, by cytofluorimetric biparametric assay. U87-MG cells were treated for 48 h with 20, 40 or 80 μM RSV and then stained with both propidium iodide and γH2AX antibody, in order to relate cell cycle phases with γH2AX expression. Fig. 2A shows the distribution of cells in different phases: RSV treatment induced a delay in cycle progression causing an accumulation of cells in S phase with a concomitant reduction in G1 and G2 phases; this effect is evident at all used doses, especially after treatment with the 80 μM dose of RSV. When biparametric analysis was performed (Fig. 2B), the pattern and percentage of γH2AX positive cell changed with RSV treatment. The 20 μM dose of RSV led to a slightly increased γH2AX expression which becomes particularly evident in S/G2 cells with a shift of the maximal signal toward S cells, after treatment with the highest doses (40 and 80 μM). On the contrary, among the cells from untreated culture there was a presence of relatively few G1 cells with γH2AX expression.

In order to check whether or not RSV could interfere with TOPO IIα activity in our experimental model, we monitored the capacity of RSV treatment to inhibit the ability of human recombinant TOPO II to decatenate kinetoplast DNA (kDNA). As showed in Fig. 3, when TOPO II is added, kDNA is cleaved and fragments are electrophoresed; on the contrary RSV addition progressively inhibits the decatenation of kDNA in a dose-related fashion. At the RSV 80 μM dose the kDNA decatenation is almost completely inhibited. To see if this behaviour could be confirmed with increasing RSV dose, we also tested the higher dose 120 μM, so obtaining a completed inhibition of kDNA decatenation.

4. Discussion

Malignant gliomas are the most common primary brain tumours in the central nervous system. Despite advances

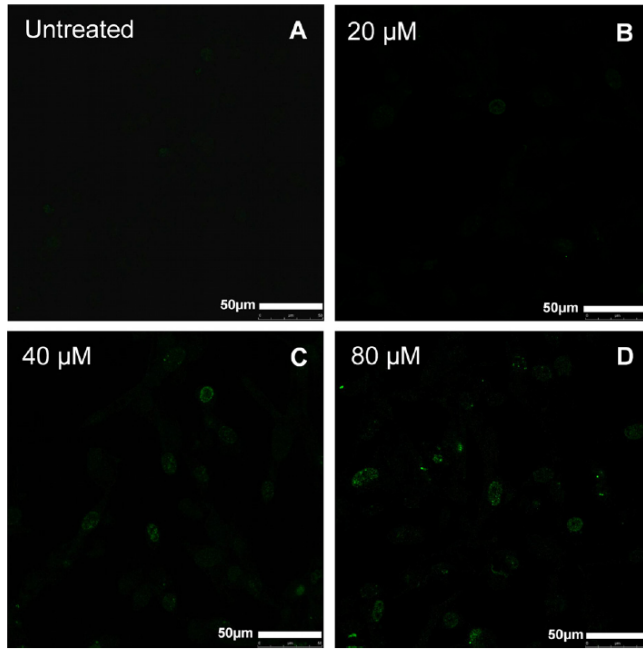

Fig. 1. Resveratrol induces the histone H2AX phosphorylation. Immunohistochemistry staining of nuclear γH2AX foci was performed with an Alexa Fluor® 488 Anti-H2AX-phosphorylated at Ser139 antibody (green) in U87-MG untreated (A) or 48 h treated with 20 µM (B), 40 µM (C) or 80 µM (D) of resveratrol. After treatment cells were fixed, permeabilized and analysed with a confocal microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in surgical intervention and radio-treatment, the prognosis of this disease remains poor. Therefore, developing novel strategies are essential in order to improve effectiveness of treatments for this disease. In recent years, many natural compounds contained in the diet have been identified as potential chemopreventive agents and/or adjuvant in conventional cancer treatment. Among them RSV, a natural product highly abundant in grapes, peanuts, red wine, has attracted research attention, owing to its cardioprotective [11], antioxidant, anti-inflammatory [12], neuroprotection activities [13] and cancer chemopreventive properties [14].

Increasing evidence shows that RSV, in addition to its chemopreventive potential, can inhibit growth, promote differentiation and/or induce apoptosis in a variety of human tumour cells, including glial cells [15–17]. The doses utilized in these studies vary widely, ranging from 10 to 250 µM. One of the concerns for using RSV is the potential toxicity at high dosages. Recently it has been reported that 100 mg/kg (body weight), currently used for studies on rodents, corresponding to a total RSV 680 µM peak serum concentration, shows no detrimental effect [4]. Several Phase I clinical trials, sponsored by The National Cancer Institute, are currently underway for oral administration in humans at doses as high as 7.5 g per day. All these data are supported by the important observation that RSV can cross the blood–brain barrier and incorporate in brain tissue after a intraperitoneal injection of a 30 mg/kg dose [18]. The most recent data show that RSV is well adsorbed, well metabolized and well tolerated with no marked toxicity [19].

With regard to these literature data, we selected three doses of RSV to study its ability in modulating cell cycle progression in human glioblastoma cells. Moreover, since after RSV treatment with the same doses we detected the presence of γH2AX foci, we analysed the RSV ability in interfering with TOPO II activity.

As regards cell cycle modulation our results are in agreement with literature and our previous data [3] on the ability of RSV to cause a delay in S phase progression with a concomitant reduction in G1 and G2 cells. In our biparametric assay, this result matched the detection of γH2AX expression, particularly evident in S phase cells.

Histone H2AX, a variant of H2A, is phosphorylated at its carboxy-terminal Serine 139 as one of the earliest cellular

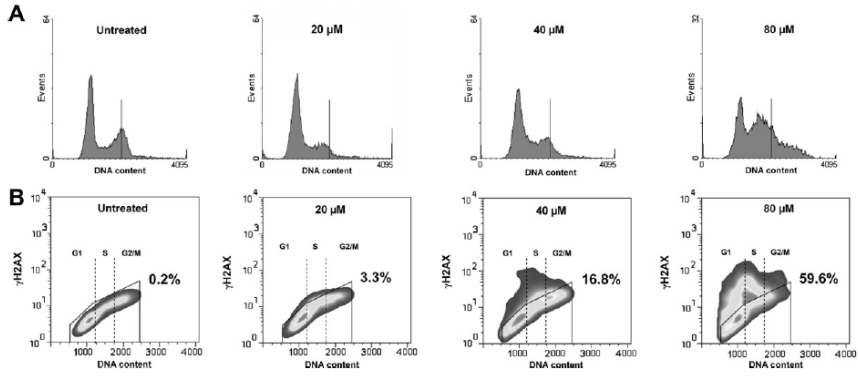

Fig. 2. Resveratrol induces cell cycle delay and histone H2AX phosphorylation in S phase. U87-MG cells in exponential growth phase were left untreated or treated for 48 h with 20 μM, 40 μM or 80 μM of resveratrol. Fixed and permeabilized cells were stained with propidium iodide (PI)/RNaseA solution for a cell cycle monoparametric analysis by flow cytometry (A). Vertical line depict a modal channel of G2/M phase cells on untreated sample. (B) At the same experimental conditions cells were treated, fixed, permeabilized and double stained with an anti-γH2AX Alexa Fluor® 488-conjugated antibody and propidium iodide/RNaseA solution. Intensity of fluorescence by γH2AX was related with cell cycle phases through a biparametric density plot analysis. The dashed lines delimits the cell cycle phases whereas the polygonal gate (exclusion gate) was depicted to evaluate the percentage of all γH2AX positive cells.

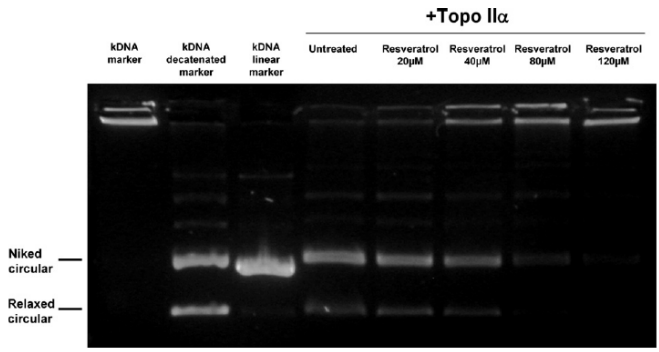

Fig. 3. Resveratrol induces the inhibition of topoisomerase IIα activity. Decatenation reactions were performed in a total volume of 20 μl and analysed on 1% agarose gel containing 0.5 μg/ml ethidium bromide. Lane 1 (left side) contained catenated kDNA alone, lanes 2 and 3 contained respectively kDNA decatenated and kDNA linear markers. Decatenation reactions contained 0.2 μg of kDNA, 4UI of human topoisomerase IIα, 20 mM ATP in presence or absence of resveratrol. The reactions was incubated at 37 °C for 1 h and terminated with a stop/loading buffer containing 5% Sarkosyl, 0.125% bromophenol blue 25%. Nicked and relaxed circular DNA represent a topoisomerase II decatenation products. kDNA catenated was retained in the well due to its large size.

response to the induction of DNA double-strand breaks (DSBs) [10]. Once phosphorylated, γH2AX foci rapidly form over chromatin regions on either side of a DSB [20] facilitating repair through relocalization and focusing factors to the site of breaks [21]. γH2AX also functions in the end-joining of DSBs by serving as an anchor for holding broken chromosomal ends in close proximity [22]. The ability of RSV in inducing DNA breaks is still debated but no evidence is present in literature about the induction of chromosomal damage. We showed a slight increase in chromosome aberrations after 200 μM RSV treatment in CHO cells and recently it has been shown that RSV is rather able to reduce radiation-induced chromosome damage *in vivo* [23].

On the other hand, the detection of a particularly high level of γH2AX expression in cells with a S DNA content suggested an influence of RSV treatment on topoisomerase activity.

Topoisomerases (TOPO) are ubiquitous enzymes which modulate the topological state of DNA in the cell, remove

knots and tangles by creating transient breaks in DNA helix. In eukaryotic cells topoisomerases type II (TOPO II) function in numerous DNA processes (recombination, proper chromosome structure and segregation) generating transient DNA DSBs [24]. Vertebrate species express two forms, α and β. TOPO IIα is essential for the survival of actively growing cells and is found at replication forks. TOPO IIβ physiological functions have yet to be defined. Beyond these functions, TOPO II is the target for some of the most active anticancer drugs currently utilized for the treatment of human cancers. These drugs are divided into two classes based on their ability to inhibit the catalytic activity of TOPO II or to increase the level of TOPO II-DNA covalent complexes (TOPO poisons). In general, agents of the first class kill cells through the elimination of the essential activities of the enzyme, while the agents in the second class convert the transient DNA–enzyme interactions to DNA DSBs [25].

We show here for the first time that RSV exerts an actual catalytic inhibition of human TOPO IIα *in vitro*. Jo et al. [26] showed that polyphenolic fractions from grape cell culture were potent inhibitor of human TOPO II, utilizing the same assay. Similar results were also achieved by Yamada et al. [27] studying four new RSV oligomers. Polyphenolic compounds belong to bioflavonoids which show a broad range of cellular activities. Among them genistein, one of the most widely consumed constituents of the human diet, has been recently shown to enhance DNA cleavage mediated by human TOPO IIα and β [28,29]. This activity is exerted by increasing the levels of DNA cleavage complexes generated by TOPO II through a direct interaction of genistein with an ATP binding motif of the enzyme.

On the whole our data suggest the possibility that RSV could act as a TOPO poison interacting *in vivo* with topoisomerase at the protein–DNA interface so increasing the lifetime of the cleavage complexes. The follow up to this hypothesis will arrive in our in progress analysis of *in vivo* TOPO II-DNA cleavage complexes and their persistence after RSV treatment, and from molecular modelling and docking techniques applied to the analysis of the three dimensional structure of RSV-DNA-topoisomerase ternary complex [30]. In fact the stability of ternary cleavage complexes and how long they remain associated is linked to the levels of DNA strand breaks induced and to the subsequent toxicity.

The confirmation of RSV as a TOPO II poison together with its ability to delay cell cycle progression could render this molecule an important anti-tumour agent against high grade gliomas which are highly resistant to most therapies. The combination of RSV and other anti-glioma therapies may be a novel strategy for the treatment of glioma that deserve further investigation.

Conflicts of interest statement

None declared.

References

[1] M. Jang, L. Cai, G.O. Udeani, K.V. Slowing, C.F. Thomas, C.W. Beecher, H.H. Fong, N.R. Farnsworth, A.D. Kinghorn, R.G. Mehta, R.C. Moon, J.M. Pezzuto, Cancer chemopreventive activity of resveratrol, a natural product derived from grapes, Science 275 (1997) 218–220.
[2] V. Cucciolla, A. Borriello, A. Oliva, P. Galletti, V. Zappia, F. Della Ragione, Resveratrol: from basic science to the clinic, Cell Cycle 6 (2007) 2495–2510.
[3] S. Leone, M. Fiore, M.G. Lauro, S. Pino, T. Cornetta, R. Cozzi, Resveratrol and X-rays affect gap junction intercellular communications in human glioblastoma cells, Mol. Carcinog. 47 (2008) 587–598.
[4] J.A. Baur, D.A. Sinclair, Therapeutic potential of resveratrol: the in vivo evidence, Nat. Rev. Drug Discov. 5 (2006) 493–506.
[5] S.A. Gatz, L. Wiesmüller, Take a break – resveratrol in action on DNA, Carcinogenesis 29 (2008) 321–332.
[6] K. Fukuhara, N. Miyata, Resveratrol as a new type of DNA-cleaving agent, Bioorg. Med. Chem. Lett. 8 (1998) 3187–3192.
[7] R. De Salvia, F. Festa, R. Ricordy, P. Perticone, R. Cozzi, Resveratrol affects in a different way primary versus fixed DNA damage induced by H(2)O(2) in mammalian cells in vitro, Toxicol. Lett. 135 (2002) 1–9.
[8] G.A. Locatelli, M. Savio, L. Forti, I. Shevelev, K. Ramadan, L.A. Stivala, V. Vannini, U. Hübscher, S. Spadari, G. Maga, Inhibition of mammalian DNA polymerases by resveratrol: mechanism and structural determinants, Biochem. J. 389 (2005) 259–268.
[9] M.P. Fuggetta, G. Lanzilli, M. Tricarico, A. Cottarelli, R. Falchetti, G. Ravagnan, E. Bonmassar, Effect of resveratrol on proliferation and telomerase activity of human colon cancer cells in vitro, J. Exp. Clin. Cancer Res. 25 (2006) 89–193.
[10] E.P. Rogakou, D.R. Pilch, A.H. Orr, V.S. Ivanova, W.M. Bonner, DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139, J. Biol. Chem. 273 (1998) 5858–5868.
[11] M. Sato, P.S. Ray, G. Maulik, N. Maulik, R.M. Engelman, A.A. Bertelli, A. Bertelli, D.K. Das, Myocardial protection with red wine extract, J. Cardiovasc. Pharmacol. 35 (2000) 263–268.
[12] Y.H. Feng, W.L. Zhou, Q.L. Wu, X.Y. Li, W.M. Zhao, J.P. Zou, Low dose of resveratrol enhanced immune response of mice, Acta Pharmacol. Sin. 23 (2002) 893–897.
[13] A. Quincozes-Santos, A.C. Andreazza, P. Nardin, C. Funchal, C.A. Gonçalves, C. Gottfried, Resveratrol attenuates oxidative-induced DNA damage in C6 Glioma cells, Neurotoxicology 28 (2007) 886–891.
[14] B.B. Aggarwal, A. Bhardwaj, R.S. Aggarwal, N.P. Seeram, S. Shishodia, Y. Takada, Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies, Anticancer Res. 24 (2004) 2783–2840.
[15] S.H. Tseng, S.M. Lin, J.C. Chen, Y.H. Su, H.Y. Huang, C.K. Chen, P.Y. Lin, Y. Chen, Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats, Clin. Cancer Res. 10 (2004) 2190–2202.
[16] H. Jiang, L. Zhang, J. Kuo, K. Kuo, S.C. Gautam, L. Groc, A.I. Rodriguez, D. Koubi, T.J. Hunter, G.B. Corcoran, M.D. Seidman, R.A. Levine, Resveratrol-induced apoptotic death in human U251 glioma cells, Mol. Cancer Ther. 4 (2005) 554–561.
[17] W. Zhang, Z. Fei, H.N. Zhen, J.N. Zhang, X. Zhang, Resveratrol inhibits cell growth and induces apoptosis of rat C6 glioma cells, J. Neurooncol. 81 (2007) 231–240.
[18] Q. Wang, J. Xu, G.E. Rottinghaus, A. Simonyi, D. Lubahn, G.Y. Sun, A.Y. Sun, Resveratrol protects against global cerebral ischemic injury in gerbils, Brain Res. 958 (2002) 439–447.
[19] C.H. Cottart, V. Nivet-Antoine, C. Laguillier-Morizot, J.L. Beaudeux, Resveratrol bioavailability and toxicity in humans, Mol. Nutr. Food Res. 54 (2010) 7–16.
[20] E.P. Rogakou, C. Boon, C. Redon, W.M. Bonner, Megabase chromatin domains involved in DNA double-strand breaks in vivo, J. Cell Biol. 146 (1999) 905–916.
[21] T.T. Paull, E.P. Rogakou, V. Yamazaki, C.U. Kirchgessner, M. Gellert, W.M. Bonner, A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage, Curr. Biol. 10 (2000) 886–895.
[22] C.H. Bassing, F.W. Alt, H2AX may function as an anchor to hold broken chromosomal DNA ends in close proximity, Cell Cycle 3 (2004) 149–153.
[23] R.E. Carsten, A.M. Bachand, S.M. Bailey, R.L. Ullrich, Resveratrol reduces radiation-induced chromosome aberration frequencies in mouse bone marrow cells, Radiat. Res. 169 (2008) 633–638.
[24] J.L. Nitiss, DNA topoisomerase II and its growing repertoire of biological functions, Nat. Rev. Cancer 9 (2009) 327–337.
[25] A.K. McClendon, N. Osheroff, DNA topoisomerase II, genotoxicity, and cancer, Mutat. Res. 623 (2007) 83–97.

[26] J.Y. Jo, E. Gonzalez de Mejia, M.A. Lila, Catalytic inhibition of human DNA topoisomerase II by interactions of grape cell culture polyphenols, J. Agric. Food Chem. 54 (2006) 2083–2087.
[27] M. Yamada, K. Hayashi, H. Hayashi, R. Tsuji, K. Kakumoto, S. Ikeda, T. Hoshino, K. Tsutsui, T. Ito, M. Iinuma, H. Nozaki, D.-G. Nepalensinols, New resveratrol oligomers from *Kobresia nepalensis* (Cyperaceae) as potent inhibitors of DNA topoisomerase II, Chem. Pharm. Bull. 54 (2006) 354–358.
[28] O.J. Bandele, N. Osheroff, Bioflavonoids as poisons of human topoisomerase II alpha and II beta, Biochemistry 46 (2007) 6097–6108.
[29] O.J. Bandele, N. Osheroff, The efficacy of topoisomerase II-targeted anticancer agents reflects the persistence of drug-induced cleavage complexes in cells, Biochemistry 47 (2008) 1900–11908.
[30] J.L. Nitiss, Targeting DNA topoisomerase II in cancer chemotherapy, Nat. Rev. Cancer 9 (2009) 338–350.

3.2 Leone, S., Basso, E., Polticelli, F. and Cozzi, R. (2012) Resveratrol acts as a topoisomerase II poison in human glioma cells. Int J Cancer, 131, E173-8.

IJC
International Journal of Cancer

Resveratrol acts as a topoisomerase II poison in human glioma cells

Stefano Leone[1], Emiliano Basso[1], Fabio Polticelli[1,2] and Renata Cozzi[1]

[1] Department of Biology, University "Roma TRE," Rome, Italy
[2] National Institute of Nuclear Physics, "Roma TRE" Section, Rome, Italy

Recently, we demonstrated that Resveratrol (RSV), a well known natural stilbene, is able to induce a delay in S progression with a concomitant increase in γH2AX expression in U87 glioma cells. Furthermore, we showed that it inhibits the ability of recombinant human topoisomerase IIα to decatenate kDNA *in vitro*. Because proliferating tumor cells express topoisomerases at high levels and these enzymes are important targets of some of the most successful anticancer drugs, we tested whether RSV is able to poison topoisomerase IIα in glioma cells. Then, we monitored the increase of micronuclei in RSV treated U87 cells as a consequence of the conversion of TOPOII/DNA cleavable complexes to permanent DNA damage. Finally, we assayed the ability of RSV in modulating the expression of target proteins involved in DNA damage signalling, namely ATR, ATM, Chk1, Chk2 and γH2AX. Through a molecular modelling here we show that RSV binds at the TOPOII/DNA interface thus establishing several hydrogen bonds. Moreover, we show that RSV poisons TOPOIIα so inducing DNA damage; ATM, Chk2 and γH2AX are involved in the DNA damage signalling after RSV treatment.

Resveratrol (3,4′,5-trihydroxylstilbene), a polyphenol synthesized by a wide variety of plant species, is well known for its antitumor potential as demonstrated by many *in vitro* studies.[1] Nevertheless, the molecular targets of Resveratrol (RSV) activity seem not yet completely understood due to the multiplicity of RSV treatment effects in normal and transformed cultured cells. However, it is well known that RSV can disturb the normal progress of the cell cycle so decreasing the proliferation and can also induce apoptosis in cell type- and concentration-dependent mode.[2] On the other hand RSV, such as many other natural polyphenols, acts as antioxidant or prooxidant due to the intracellular presence of transition metal ions. The prooxidant activity could be an important action mechanism for its anticancer properties.[3]

It has been suggested that some of the cellular effects of polyphenols, such as anti-proliferative and proapoptotic actions, could be correlated with their ability to act on topoisomerases.[4,5] These are ubiquitous nuclear enzymes that modulate the topological state of DNA by breaking and resealing one or both strands of a DNA duplex. In eukaryotic cells, type II topoisomerases, isoforms α and β, function during major cellular processes involving DNA (recombination, replication, proper chromosome structure and segregation) generating intermediate cleavable or covalent complexes with a short half-life.[6] Highly proliferating tumor cells express these enzymes, particularly Type IIα, at levels many times higher than quiescent cells [7,8] so that topoisomerases II are important targets for some of the anticancer drugs most successfully used in the treatment of human malignancies. TOPOII-targeted agents interfere with the binding between DNA and TOPOII or act by increasing the concentration of topoisomerase–DNA covalent complexes. These agents shifting the equilibrium of the cleavage/religation reaction can provoke permanent DNA double strand breaks (DSBs) triggering cell death and/or causing chromosomal aberrations.[9,10] Hence the need to search for new anticancer drugs able to poison TOPOII in proliferating cells and showing a moderate cytotoxic potential in quiescent ones.

Recently, we have shown that RSV treatment is able to induce a delay in S progression with a concomitant increase in γH2AX expression in U87 glioma cells.[11] Furthermore, in an *in vitro* assay, RSV was shown to inhibit the ability of recombinant human TOPOIIα to decatenate kDNA.

Previously, Yamada *et al.*,[12] reported similar results studying RSV oligomers.

Since other polyphenols, namely bioflavonoid as genistein, have been shown to enhance DNA cleavage mediated by human TOPOII [5,13] we tested the hypothesis that RSV could act as a TOPO poison. We first focused our attention on the interference of RSV molecule between DNA and topoisomerase II with a molecular modelling study. Once the possibility of this molecular interaction was verified, we analyzed through the *in vivo* complex of enzyme (ICE) assay the induction of stabilized cleavable complexes between DNA and TOPOIIα after RSV treatment in U87 cells. We next monitored the increase of micronuclei (MN) in RSV-treated U87 cells as a consequence of the conversion of TOPOII/

Key words: DNA/TOPO cleavable complex, docking simulation, micronuclei, DNA damage signalling
DOI: 10.1002/ijc.27358
History: Received 12 Jul 2011; Accepted 4 Nov 2011; Online 18 Nov 2011
Correspondence to: Renata Cozzi, Viale G. Marconi 446, Roma 00146, Italy, Tel.: +39-6-57336330, Fax: +39-6-57336321, E-mail: cozzi@uniroma3.it

DNA complexes to permanent DNA damage. Finally, RSV was assayed for its ability in modulating the expression of target proteins involved in damage signalling namely ATR, ATM, Chk1, Chk2 and γH2AX.

Material and Methods

Cell culture and reagents

U87-MG glioblastoma cells were maintained in DMEM supplemented with inactivated 10% fetal bovine serum, 5mM L-glutamine and gentamicin and incubated at 37°C (5% CO_2). All chemicals were purchased from Sigma Aldrich (St. Louis, MO) except antibodies (Cell signalling tech., Beverly, MA) and ICE assay kit (Topogen, Port Orange, FL).

Modelling analysis

Docking simulations of RSV onto TOPOIIα–DNA complex (PDB code: 2RGR)[14] were performed using PatchDock,[15] a molecular rigid-body docking algorithm based on shape complementarity principles. The 20 best ranking complexes, according to PatchDock scoring function, were visually analyzed and the complex displaying the highest number of molecular interactions was chosen and displayed.

Analysis of TOPOII/DNA cleavable complex formation (ICE assay)

The ICE assay was used to monitor protein–DNA complexes in cells.[16] 1×10^7 U87-MG cells were treated for 30 min with RSV (120 μM) and VP-16 (positive control) (100 μM), quickly lysed with 1 mL of 1% sarkosyl. Two milliliters of each CsCl solution (1.82, 1.72, 1.50 and 1.37 g/mL) were layered successively in a polyallomer tube to generate CsCl gradient. Lysate was stratified over discontinuous CsCl gradient and then ultracentrifuged (31,000 rpm) at room temperature for 24 hr in a Beckman SW41 rotor. Twenty fractions for sample were collected and absorbance values at 260 nm were determined. DNA positive fractions were spotted on nitrocellulose by a slot blot apparatus. TopoII/DNA complexes were immunodetected with anti-TopoII antibody conjugated with an anti-rabbit-HRP secondary antibody and finally revealed with ECL.

Cytokinesis block micronucleus assay

10^5 cells for each experimental point were seeded on coverslips in 35-mm Petri dish. Cells were treated with RSV at the final concentrations of 40, 80 and 120 μM for 24 or 30 hr in the presence of cytochalasin B (2 μg/mL) to arrest cytokinesis. The treatment with VP-16 (10 μM) lasted 4 hr followed by further 20 or 26 hr in presence of cytochalasin B. Then, cells were treated with hypotonic solution (KCl 0.075 M) for 2 min and fixed with absolute methanol for 10 min. Cells were stained with conventional Giemsa method and analyzed under optical microscope. For each experimental point, 1,000 binucleated cells were analyzed and were counted for total MN.[17] The data are expressed as yield of MN (*i.e.*, the total number of MN per 1,000 binucleated cells). Cell proliferation has been 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.

Western blot analysis

Cells were treated for 24 hr with 20, 40 or 80 μM of RSV, or with VP16 (10 μM) as positive control. Only for γH2AX analysis, cell cultures were washed after RSV treatment and immediately pelleted (*t*0) or recovered after further 24 hr (*t*24). After washing, cells were solubilized with lysis buffer (0.5 M Tris-HCl (pH 6.8), 2% SDS, 30% glycerol, 100 mM 2-β mercaptoethanol) and boiled for 5 min. Equal amounts of whole protein lysate (20 μg) were loaded and separated on 8–16% gradient (Nusep precast Longlife gel), transferred on nitrocellulose, incubated with antibodies; signals were revealed by autoradiography using the ECL detection kit (Pierce, Rockford, IL).[18]

Primary antibodies used were anti-phospho-Chk1 (Ser296), anti-phospho-Chk2, anti-phospho-ATM (S1981), anti-phospho-ATR (S428), anti-γH2A.X (S139) and anti-α tubulin; secondary antibody is an anti-mouse or rabbit IgG, HRP-linked (Cell signalling tech.).

Results

Molecular interaction of RSV with DNA and TOPOII

The possibility of a direct interaction between RSV and TOPOII was first investigated *in silico* using the rigid-body docking algorithm implemented in PatchDock.[15] PatchDock analysis returned 20 stereochemically feasible RSV–TOPOII complexes which were further analyzed in terms of the number of hydrogen bonds. The best complex resulting from this latter analysis is shown in Figure 1. According to PatchDock scoring function, the potential candidate we chose is ranked second in interface area of the complex, fifth in atomic contact energy and sixth in geometric shape complementarity. In this complex, RSV binds at the TOPOII–DNA interface establishing several hydrogen bonds with both the protein groups and DNA bases and sugars. In detail, one of the hydroxyl groups of RSV is hydrogen bonded to the backbone carbonyl of Pro969 while the other two hydroxyl groups form hydrogen bonds with the side-chain hydroxyl group of Ser838, with one of the DNA sugar oxygen (T10 O4′), and with the N2 atom of G9 and the O2 atom of C11. The binding mode observed in docking simulations indicates that RSV could stabilize the TOPOII/DNA complex by "cross-linking," mainly through bridging hydrogen bonds, the protein moiety to DNA.

Stabilization of cleavable DNA/TOPOII complexes

The result obtained with molecular modelling suggests the hypothesis that RSV can interact with both DNA and TOPOII molecules to form non-covalent complexes thus affecting the cleavage/religation equilibrium. In general, drugs targeting TOPOII act impairing the ability of the enzyme to religate cleaved DNA or enhancing the forward rate of DNA cleavable complex formation.[14] The important biological consequence is the stabilization of cleavable complexes.

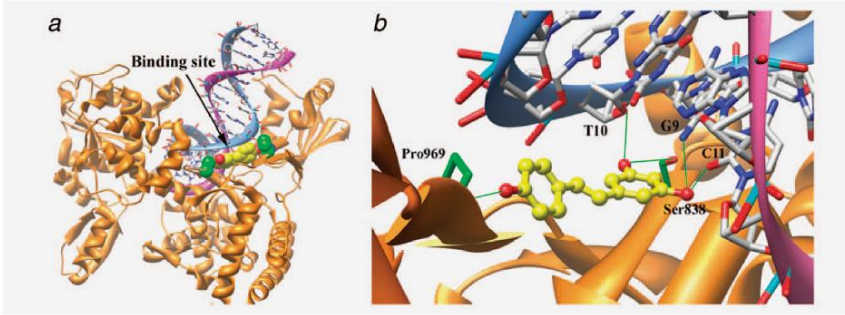

Figure 1. Schematic representation of the topoisomerase IIα–DNA–resveratrol putative complex obtained by docking simulations. (*a*) View of the topoisomerase IIα–DNA complex (monomer) showing the resveratrol binding site (arrow). For clarity, only DNA, resveratrol and the protein groups interacting with it are shown. Resveratrol and protein carbon atoms are shown in yellow and green, respectively. (*b*) Detail of the molecular interactions between resveratrol, protein and DNA. Part of the topoisomerase backbone has been removed to better show resveratrol binding site. Hydrogen bonds are represented by green lines.

To test weather RSV is able to enhance the stability of TOPOII/DNA complexes, we used an ICE[16] bioassay widely used in tissue culture and tumor samples.[16,19] As positive control was used VP-16, a specific TOPOII poison was able to leave topoisomerase covalently bound to the 5′-phosphate bond thus freezing the cleaved complex.[20]

After CsCl gradient separation, four DNA positive fractions (16–19) were blotted and probed with anti-TopoII antibody. The image presented in Figure 2 clearly indicates that topoisomerase II is present in the DNA containing fractions from RSV treated cells as well as in VP-16 treated ones. On the contrary in untreated samples, a slight physiological immuno-positivity for TOPOIIα is present. These results demonstrate, for the first time, that RSV enhances cleavable TOPOII/DNA complexes.

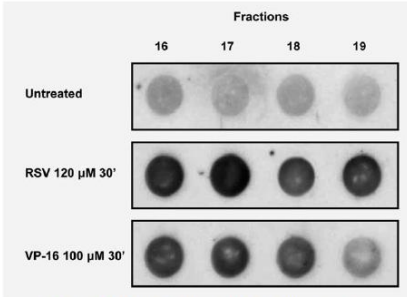

Figure 2. TOPOII/DNA cleavable complex formation (ICE assay). U87 cells were treated for 30 min with RSV or VP-16 and lysates were centrifuged over discontinuous CsCl gradient. TOPOII/DNA complexes were immunodetected with anti-TopoII antibody. The DNA positive fractions probed with anti-TopoII antibody are shown.

Micronuclei induction

Since the stabilized cleavable complexes can represent an obstacle for the progression of the replication fork leading to the formation of DNA DSBs,[21] we examined the induction of DNA damage by RSV treatment in U87 cells through micronucleus assay. MN provide a convenient and reliable index of both chromosome breakage and loss. They are present in cells that have completed nuclear division so that are scored in the binucleated stage of cell cycle.[22]

In Figure 3, we show that RSV treatment induces a slight but significant increase ($p < 0.05$) of MN in a dose-dependent manner although less efficiently than VP-16. In fact the highest dose of RSV (120 μM) causes a six-fold increase of baseline MN, while VP-16, a well-known inducer of MN,[23] appears to be more efficient ($p < 0.001$) than RSV also when used at a lower dose (10 μM).

As far as NDI, it ranges from 1.76 in control cells to 1.14 at the highest dose of RSV, showing a reduction of 35% of cell proliferation. The NDI after 10 μM VP16 treatment was 1.3.

ATM, ATR, Chk1, Chk2 and γH2AX expression

We then examined whether the DNA damage induced by RSV treatment could lead to the activation of ATM/ATR damage signalling pathways. As shown in Figure 4*a*, 80 μM RSV increases the level of the activated ATM (P-Ser1981) and consequently increases the activated Chk2 (P-Thr68). Furthermore, since H2AX is one of the substrates phosphorylated by ATM and concomitant activation of ATM and H2AX phosphorylation is considered as a reporter of DSBs, we analyzed the expression of γH2AX (P-Ser139), immediately after RSV or VP-16 treatment and after a 24 hr-recovery time. We observed an increase of γH2AX level at Time 0 that persists at Time 24 (Fig. 4*b*). As far as the activation of ATR and its downstream kinase Chk1, surprisingly we did

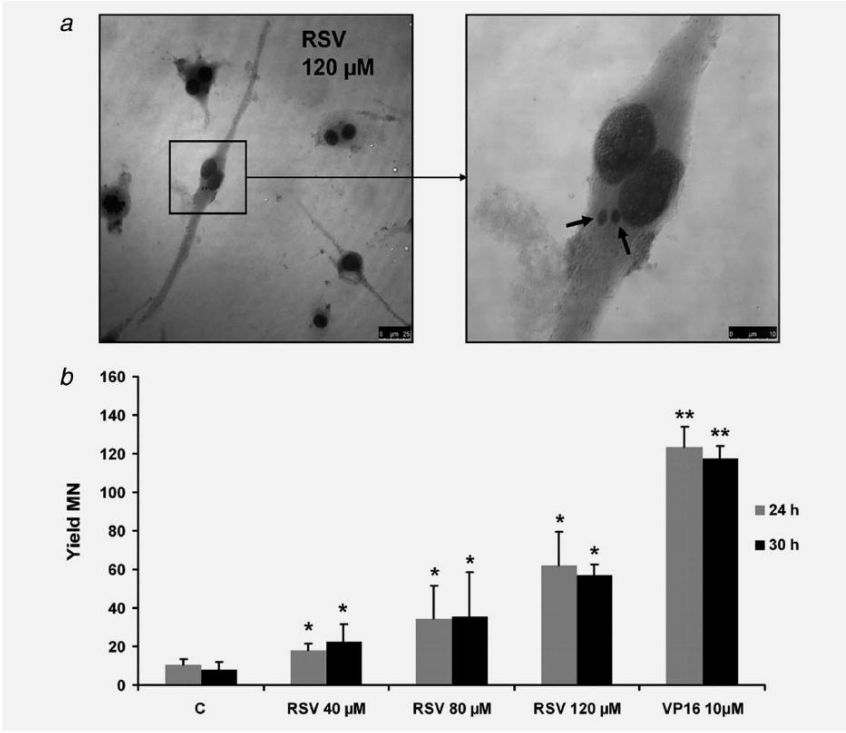

Figure 3. Micronuclei induction following resveratrol and VP-16 treatment in U87 cells. The treatment with RSV lasted 24 and 30 hr; the treatment with VP-16 lasted 4 hr followed by 20 and 26 hr of recovery time, respectively. (*a*) Binucleated cells obtained after RSV treatment. The arrows show a micronucleus. (*b*) Values represented as bar graphs are the mean of three independent experiments ± SD. $*p < 0.05$, $**p < 0.01$ at Mann-Whitney U-test.

not observe any increase in the phosphorylated form of ATR (P-Ser428) and just a slight increase in Chk1 (P-Ser296) level at the highest RSV dose (Fig. 4*c*).

Discussion

Extensive *in vitro* studies revealed multiple intracellular targets of RSV, which affect cell proliferation and death. On the other hand much is known about RSV anti oxidant properties exerted as scavenger of free radicals or as promoter of the activities of antioxidant enzymes. However RSV acts also as proooxidant depending on concentration and cell type and this effect is due to the presence of transition metal ions. Since cancer cells (particularly glioma cells) contain elevated levels of copper compared to normal ones,[24] RSV can act more efficiently in killing them. A particular chronic treatment with RSV can induce an increased level of reactive oxygen species together with a delay in cell cycle progression in cancer cells.[25] These data are confirmed by many authors using doses comparable to those used in our article.[26] The pro-oxidant action which involves the mobilization of endogenous copper could be a common mechanism for anticancer and chemopreventive properties of plant polyphenols. These results are also consistent with what is well known about other polyphenols which have both anti- and pro-oxidant activity[27] and inhibit topoisomerase II activity depending on concentrations, duration and mode of treatment, cell types. This is the case of genistein, *e.g.*, example that shows both pro-oxidant[28] and anti-topoisomerase activity.[13]

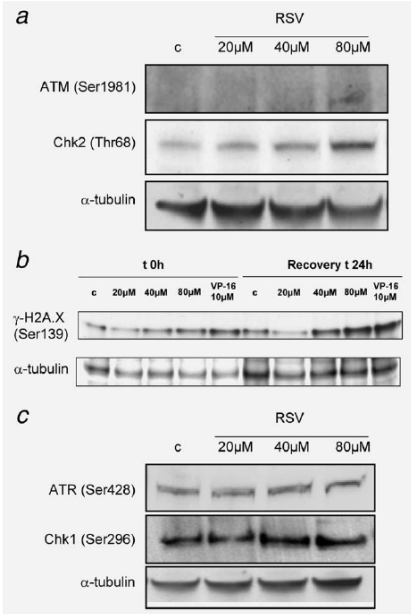

Figure 4. Effect of resveratrol treatment on the expression of DNA damage signalling proteins. U87 cells were treated with 20–40–80 μM RSV for 24 hr. (*a*) ATM and Chk2 expression; (*b*) γH2AX expression evaluated immediately after RSV or VP-16 treatment (*t*0) and after a 24 hr recovery period (*t*24); (*c*) ATR and Chk1 expression.

Our group previously focused on the effect of RSV treatment on human glioma cells *in vitro* showing its ability to induce a delay during S phase progression together with the improvement of intercellular junctions.[11,18] Furthermore, we showed its ability in inhibiting in an *in vitro* test topoisomerase IIα catalytic activity.[11]

Topoisomerase II is the primary target for some of the most active drugs currently in use for the treatment of human cancers.[29] Among these agents, the so called TOPO-poisons increase the levels of enzyme–DNA cleavable complexes by interacting with topoisomerase II at the protein–DNA interface in a non-covalent manner or covalently modifying the structure of the protein and/or the DNA.[30] In this context, the genotoxic activity of some human dietary components such as bioflavonoid has been attributed to their action as topoisomerase poisons.[5]

In this study, we demonstrate through docking simulations that RSV polar groups allow this molecule to establish non-covalent cross-linking interactions with both TOPOII and DNA at the binding interface between these two macromolecules. This result is particularly interesting in that it is suggestive of a stabilizing effect of RSV on the TOPOII/DNA complex which, in turn, could cause a delay in DNA religation. Thus, our next target was to test the stabilization of cleavable complexes after RSV treatment. Under our experimental condition, RSV and VP-16 show the same ability to induce cleavage complexes in our cells, while their effect on the generation of permanent DNA damage was not equal. In fact when we analyzed MN, VP-16 proved to be about 20 times more active. This difference might correlate with the persistence of cleavage complexes formed during treatment. It is known that VP-16 induces more stable complexes than genistein[13] and this stability may determine the likelihood that the complexes could be converted in permanent damage, such as MN. In this study, we did not analyze the persistence of RSV-induced complexes and we do not know whether the specificity of RSV binding with DNA sites could influence this aspect. However, differences observed between the effects caused by RSV and VP-16 treatment could be due to the non-covalent nature of the RSV-TOPOII–DNA ternary complex which would only delay the DNA religation process and not leave topoisomerase covalently bound to DNA, as observed with VP-16.[20] This issue deserves to be investigated in deeper detail to better understand the long-term cytotoxicity of RSV treatment.

The induction of DSBs through TOPOII poisoning by RSV treatment is also confirmed by γH2AX expression immediately after treatment and by its persistence after a recovery period. These data together with the increase in the phosphorylated form of ATM and Chk2 expression lead us to conclude that RSV induced DNA damage is sensed by early signal transducers that activate S phase arrest. These data are in agreement with what shown by Tyagi *et al.*[31] and ourselves on S phase delay induced by RSV treatment in U87 cells.[11] However, the ability of topoisomerase inhibitors to activate the signalling cascade through ATM pathway is well documented for etoposide.[32]

As far as the ATR/Chk1 pathway is concerned, the lack of any increase in the expression of P-ATR is quite surprising. In fact the phosphorylated forms of both kinases ATR (Ser428) and Chk1 (Ser296) are present in basal conditions and only Chk1 is slightly increased by RSV treatment.

This is in contrast with the results obtained by Tyagi *et al.*[31] in a human ovarian carcinoma model. They showed an increase in ATR with a consequent increase in phosphorylation of Chk1 as well as in total protein level after RSV treatment. Topoisomerase catalytic inhibitors, such as ICRF, that do not stabilize the cleavable complex are also known to induce the ATR/Chk1 pathway.[33]

It is known that the full activation of the ATR pathway requires the localization of ATR–ATRIP complex to sites of DNA damage and stressed replication forks, and the stimulation of this complex by its regulator TopBP1.[34] The nature of this interaction would allow ATR to undergo activation in a very

dynamic[35] and substantially different manner from that of ATM, which involves autophosphorylation at Ser1981.[36] Thus, it could be that the increase in ATR activation occurs at an earlier time with respect to the time interval analyzed in this report.

On the whole, our results strongly support the idea that RSV poisons TOPOIIα so inducing DNA damage and that ATM, Chk2 and γH2AX are involved in the DNA damage signalling after RSV treatment. These results also suggest that the type of DNA damage induced by RSV might involve DSBs even if it must be taken into account that the most prominent phenotypes observed after blocking of TOPOII function are defects in chromatid decatenation and segregation resulting in chromatid breakage and non-disjunction. Thereby future studies should focus on the possible evolution of DNA damage induced by RSV into chromosome breaks and/or polyploidy and endoreduplication. These aberrant cells are bound to cell death due to mitotic catastrophe or to induction of apoptosis.

In our opinion, our overall results highlight a new mechanism of action of RSV that together with its known multiple biological activities may provide new insights into the potential role of RSV as an anticancer drug *per se* and also as modulator of the cytotoxic effects of other anticancer agents routinely used in the therapy of particularly resistant tumors, such as gliomas.

References

1. Gatz SA, Wiesmuller L. Take a break—resveratrol in action on DNA. *Carcinogenesis* 2008;29: 321–32.
2. Kraft TE, Parisotto D, Schempp C, Efferth T. Fighting cancer with red wine? Molecular mechanisms of resveratrol. *Crit Rev Food Sci Nutr* 2009;49:782–99.
3. Zheng LF, Wei QY, Cai YJ, Fang JG, Zhou B, Yang L, Liu ZL. DNA damage induced by resveratrol and its synthetic analogues in the presence of Cu (II) ions: mechanism and structure-activity relationship. *Free Radic Biol Med* 2006;41:1807–16.
4. Snyder RD, Gillies PJ. Evaluation of the clastogenic, DNA intercalative, and topoisomerase II-interactive properties of bioflavonoids in Chinese hamster V79 cells. *Environ Mol Mutagen* 2002;40:266–76.
5. Bandele OJ, Osheroff N. Bioflavonoids as poisons of human topoisomerase II alpha and II beta. *Biochemistry* 2007;46:6097–108.
6. Nitiss JL. DNA topoisomerase II and its growing repertoire of biological functions. *Nat Rev Cancer* 2009;9:327–37.
7. Heck MM, Earnshaw WC. Topoisomerase II: A specific marker for cell proliferation. *J Cell Biol* 1986;103:2569–81.
8. Hsiang YH, Wu HY, Liu LF. Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. *Cancer Res* 1988;48:3230–5.
9. Larsen AK, Escargueil AE, Skladanowski A. Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol Ther* 2003;99:167–81.
10. Felix CA. Leukemias related to treatment with DNA topoisomerase II inhibitors. *Med Pediatr Oncol* 2001;36:525–35.
11. Leone S, Cornetta T, Basso E, Cozzi R. Resveratrol induces DNA double-strand breaks through human topoisomerase II interaction. *Cancer Lett* 2010;295:167–72.
12. Yamada M, Hayashi K, Hayashi H, Tsuji R, Kakumoto K, Ikeda S, Hoshino T, Tsutsui K, Ito T, Iinuma M, Nozaki H. Nepalensinols D-G, new resveratrol oligomers from Kobresia nepalensis (Cyperaceae) as potent inhibitors of DNA topoisomerase II. *Chem Pharm Bull* 2006;54:354–8.
13. Bandele OJ, Osheroff N. The efficacy of topoisomerase II-targeted anticancer agents reflects the persistence of drug-induced cleavage complexes in cells. *Biochemistry* 2008;47:11900–8.
14. Dong KC, Berger JM. Structural basis for gate-DNA recognition and bending by type IIA topoisomerases. *Nature* 2007;450:1201–5.
15. Schneidman-Duhovny D, Inbar Y, Polak V, Shatsky M, Halperin I, Benyamini H, Barzilai A, Dror O, Haspel N, Nussinov R, Wolfson HJ. Taking geometry to its edge: fast unbound rigid (and hinge-bent) docking. *Proteins* 2003;52:107–12.
16. Subramanian D, Kraut E, Staubus A, Young DC, Muller MT. Analysis of topoisomerase I/DNA complexes in patients administered topotecan. *Cancer Res* 1995;55:2097–103.
17. Fenech M. The in vitro micronucleus technique. *Mutat Res* 2000;455:81–95.
18. Leone S, Fiore M, Lauro MG, Pino S, Cornetta T, Cozzi R. Resveratrol and X rays affect gap junction intercellular communications in human glioblastoma cells. *Mol Carcinog* 2008;47:587–98.
19. Subramanian D, Furbee CS, Muller MT. ICE bioassay. Isolating in vivo complexes of enzyme to DNA. *Methods Mol Biol* 2001;95:137–47.
20. Henwood JM, Brogden RN. Etoposide. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in combination chemotherapy of cancer. *Drugs* 1990;39:438–90.
21. Michel B, Ehrlich SD, Uzest M. DNA double-strand breaks caused by replication arrest. *EMBO J* 1997;16:430–8.
22. Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD, Thomas P. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis* 2011;26:125–32.
23. Bryce SM, Bemis JC, Avlasevich SL, Dertinger SD. In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. *Mutat Res* 2007;630:78–91.
24. Gagliano N, Aldini G, Colombo G, Rossi R, Colombo R, Gioia M, Milzani A, Dalle-Donne I. The potential of resveratrol against human gliomas. *Anticancer Drugs* 2010;21:140–50.
25. Heiss EH, Schilder YD, Dirsch VM. Chronic treatment with resveratrol induces redox stress-and ataxia telangiectasia-mutated (ATM)-dependent senescence in p53-positive cancer cells. *J Biol Chem* 2007;282:26759–66.
26. Azmi AS, Bhat SH, Hanif S, Hadi SM. Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: a putative mechanism for anticancer properties. *FEBS Lett* 2006;580:533–8.
27. Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic Biol Med* 1997;22:749–60.
28. Sánchez Y, Calle C, de Blas E, Aller P. Modulation of arsenic trioxide-induced apoptosis by genistein and functionally related agents in U937 human leukaemia cells. Regulation by ROS and mitogen-activated protein kinases. *Chem Biol Interact* 2009;182:37–44.
29. Corbett AH, Osheroff N. When good enzymes go bad: conversion of topoisomerase II to a cellular toxin by antineoplastic drugs. *Chem Res Toxicol* 1993;6:585–97.
30. McClendon AK, Osheroff N. DNA topoisomerase II, genotoxicity, and cancer. *Mutat Res* 2007;623: 83–97.
31. Tyagi A, Singh RP, Agarwal C, Siriwardana S, Sclafani RA, Agarwal R. Resveratrol causes Cdc2-tyr15 phosphorylation via ATM/ATR-Chk1/2-Cdc25C pathway as a central mechanism for S phase arrest in human ovarian carcinoma Ovcar-3 cells. *Carcinogenesis* 2005;26:1978–87.
32. Tanaka T, Halicka HD, Traganos F, Seiter K, Darzynkiewicz Z. Induction of ATM activation, histone H2AX phosphorylation and apoptosis by etoposide: relation to cell cycle phase. *Cell Cycle* 2007;6:371–6.
33. Park I, Avraham HK. Cell cycle-dependent DNA damage signaling induced by ICRF-193 involves ATM, ATR, CHK2, and BRCA1. *Exp Cell Res* 2006;312:1996–2008.
34. Kumagai A, Dunphy WG. How cells activate ATR. *Cell Cycle* 2006;5:1265–8.
35. Kumagai A, Lee J, Yoo HY, Dunphy WG. TopBP1 activates the ATR-ATRIP complex. *Cell* 2006;124:943–55.
36. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003;421:499–506.

3.3 Basso, E., Fiore, M., Leone, S., Degrassi, F. and Cozzi, R. (2012) Effects of Resveratrol on Topoisomerase II-α activity: induction of micronuclei and inhibition of chromosome egregation in CHO-K1 cells. Mutagenesis (in press).

Mutagenesis pp. 1–6, 2012 doi:10.1093/mutage/ges067

Effects of resveratrol on topoisomerase II-α activity: induction of micronuclei and inhibition of chromosome segregation in CHO-K1 cells

Emiliano Basso[1], Mario Fiore[2], Stefano Leone[1], Francesca Degrassi[2] and RenataCozzi[1,*]

[1]Dipartimento di Biologia, Università "Roma TRE", Roma, Italia, [2]Istituto di Biologia Molecolare e Patologia CNR, Roma, Italia

*To whom correspondence should be addressed. Tel: +39 6 57336330; Fax: +39-6-57336321; Email: cozzi@uniroma3.it

Received on March 21 2012; revised on 8 29 2012; accepted October 1 2012

In the last years, a great interest has emerged on the resveratrol (RSV) activity in the prevention of various pathologies including cancer. We recently showed that RSV is able to interfere with topoisomerase II-α (TOPO2) activity in cancer cells, thus inducing a delay in S-phase progression with a concomitant phosphorylation of the histone H2AX. TOPO2 is mainly active in proliferating cells and is involved in the resolution of supercoiled DNA and chromosome segregation during mitosis. Here, we studied the effects of RSV in CHO-K1 cells concerning to chromosome damage and segregation as a consequence of TOPO2 inhibition. We show an increase in micronuclei and in polyploid and endoreduplicated cells due to an incorrect chromosome segregation. Furthermore, since the incomplete segregation can also affect the normal distribution of mitotic figures, we checked mitosis progression showing an increase in metaphases in relation to ana-telophases after RSV treatment. On the whole, our data show that RSV affects chromosome stability and segregation in proliferating cells probably interfering with TOPO2 activity.

Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) (RSV) is a polyphenol found in grapes (*Vitis vinifera*), berries and peanuts and in medicinal plants, such as Japanese knotweed (*Polygonum cuspidatum*) (1).

Scientific interest in RSV has continually grown since 1997, when it was first demonstrated to prevent carcinogenesis in mice (2). In the last years, this molecule has received considerable attention for its anti-inflammatory, anti-tumourigenic and anti-oxidant properties, as well as its ability to increase lifespan in lower organisms and improve general health in mammals (3). Particular attention has grown relatively to RSV ability in interfering with carcinogenic process, thus suggesting the potential use both as chemopreventive and therapeutic agent. In particular, many *in vitro* studies have been focused on anti-proliferative and pro-apoptotic effects through the activation of many intracellular targets such as tumour suppressors and cell cycle regulators (4). These actions together with the ability in activating DNA damage response via ATM/ATR are in line with the concept of RSV as a cancer chemopreventive agent (5,6). However, despite the large amount of data present in literature, several aspects of its mechanism of action remain unclear. Some clinical trials of either supplemented or dietary (e.g. grapes, grape juice, peanut butter) RSV are currently at various stages of completion. These trials are focused on resveratrol's ability in producing beneficial effects on health and also on cancer patients as target population (7).

We have previously reported that RSV treatment of human glioblastoma cells induces a delay in cell cycle progression during S phase associated with an increase in histone H2AX phosphorylation (γH2AX) (8) that is a hallmark of DNA double strand breaks (DSBs) (9). Furthermore, with an *in vitro* assay of topoisomerase II-α (TOPO2) catalytic activity we showed that RSV is able to inhibit the ability of recombinant human TOPO2 to decatenate kDNA, so that it could be considered a TOPO2 inhibitor (8). Previously, Jo *et al*. (10) showed that polyphenolic fractions from grape cell culture are potent inhibitor of human TOPO2, utilising the same assay.

Recently, through a molecular modelling we showed that RSV binds at the TOPO2/DNA interface establishing several hydrogen bonds and as a result of this interaction it is able to stabilise cleavable complexes between DNA and TOPO2. We also found an increase of DSBs as measured through micronuclei analysis confirmed by an increase in the expression of phosphorylated form of ATM, Chk2 and H2AX (11).

TOPO2 is an essential enzyme maximally expressed in G_2 and M phases of the cell cycle because it is involved in the final stages of DNA replication to facilitate chromosome untangling, condensation and mitotic segregation (12,13).

Thus, TOPO2 can separate knotted and intertwined DNA molecules and its activity is required for chromosome condensation, decatenation of intertwined daughter DNA duplexes and centromere resolution (14,15). DNA and TOPO2 form a reversible, covalent complex, often referred to as the cleavage complex (13,16).

The levels of TOPO2 protein are particularly high in cancer cells due to their high proliferative rate and this behaviour has made TOPO2 the primary cellular target for a number of widely used antineoplastic drugs. In general, TOPO2 inhibitors are divided into two classes: poisons and catalytic inhibitors. TOPO2 poisons stabilise the cleavage complex, which may block DNA replication forks or transcriptional machinery and create DSBs (17). Catalytic inhibitors are a heterogeneous group of compounds that might interfere with the binding between DNA and the enzyme, stabilise non-covalent DNA-TOPO2 complexes or inhibit ATP binding (18).

Studies using TOPO2 catalytic inhibitors suggest that G_2 cells monitor the catenation state of intertwined sister chromatids following DNA replication and actively delay progression into mitosis pending sufficient chromatid decatenation (19).

In this study, we analysed the effects of RSV treatment on the maintenance of genomic stability and chromosome segregation in Chinese Hamster Ovary (CHO) K1 cells. We performed the cytokinesis-block micronucleus assay (CBMN) accompanied by anti-kinetochore antibody staining (CREST staining) in order to investigate the induction of micronuclei containing or not containing a centromere (20). Furthermore, as it has been demonstrated that the clastogenic effect of a topo-poison could be reduced by the concomitant action of a topoisomerase inhibitor (21), we analysed the effect of combined treatment

RSV plus ethidium bromide. Then we analysed the effects on chromosome segregation by detecting the presence of polyploid (POL) or endoreduplicated (ENDO) metaphases. Endoreduplication consists of two successive rounds of DNA replication without an intervening mitosis. The visible mitotic manifestation of previous endoreduplication is the presence of diplochromosomes, made up of four chromatids held together, instead of the two normally observed in metaphase chromosomes (22). Finally, we checked the presence of anaphase bridges during mitosis as a consequence of an incomplete decatenation (23).

Materials and methods

Cell culture and treatments

CHO-K1 cells 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_2. All reagents were purchased from Euroclone (Milan, Italy). Further, 10^5 cells were seeded on coverslips in 35-mm Petri dishes the day before the experiment for CBMN, mitosis analysis, immunofluorescence and anti-kinetochore antibody staining (CREST). Next, 3×10^5 cells were seeded in 60-mm Petri dish the day before the experiment for metaphase preparation. Cells were treated with RSV (Sigma, St Louis, MO, USA) 40, 80 and 120 µM for 6 h in CBMN and metaphase preparation experiments, followed by a recovery time of 22 h. Furthermore, in CBMN experiments, we also performed the combined treatments RSV + 50 µM ethidiun bromide (EtBr). In CREST experiments, we utilised the same doses of RSV but treatments lasted 2 h followed by a recovery time of 4 h in presence of cytochalasin B.

The same RSV doses were utilised in mitosis and immunofluorescence experiments and treatments lasted 1 or 3 h without any recovery time.

Cytokinesis-block micronucleus assay

The recovery time after treatment was carried out in presence of 2 µg/ml cytochalasin B (Sigma, St Louis, MO, USA) in order to obtain binucleated cells. Cells were fixed in cold methanol for 10 min, allowed to air dry and stained with conventional Giemsa method. For each experimental point, at least 1000 binucleated cells (BN) 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 × M1 + 2 × M2 + 3 × M3 + 4 × 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.

Metaphase preparation

In order to obtain a sufficient number of metaphase spreads, colcemid (Sigma, St Louis, MO, USA; 0.1 µg/ml) was added to each culture 2 h before fixing. After hypotonic treatment (0.075M KCl) lasting 10 min at 37°C, the cells were treated three times with freshly prepared methanol, glacial acetic acid 3:1 fixative. The fixed cells were dropped onto glass slides, allowed to air dry and stained with conventional Giemsa method. The frequency of POL and ENDO cells was analysed in at least 1000 metaphases containing not less than 21 ± 1 chromosomes for each experimental point (POL‰ and ENDO‰). We considered as POL cells those owning a triploid (3n) or higher karyotype and as ENDO cells those showing all chromosomes as diplochromosomes. The images were captured and analysed using a Leica TCS SP5 laser scanning confocal microscope controlled by the LAS AF software.

Analysis of mitotic progression

After treatment, cells were treated two times in methanol:glacial acetic acid 3:1 fixative for 10 min and stained with conventional Giemsa method. For each experimental point, at least 200 mitosis were analysed and the frequency of prophases, prometa/metaphases and ana-telophases was scored. The frequency of anaphase bridges was detected in at least 100 anaphases.

Immunofluorescence and anti-kinetochore antibody staining (CREST staining)

Cells were rinsed in PHEM (60 mM PIPES, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$) fixed for 10 min with 3.7% formaldehyde in PHEM and permeabilised 5 min in 0.2% Triton X-100. Cells were blocked in phosphate-buffered saline containing 20% goat serum for 30 min at 37°C, before being processed for immunofluorescence. Antibody dilution was as follows: human anti-KT serum (CREST, Antibodies Inc., Davis, CA; 1:50) and FITC-anti-α-tubulin (Sigma, St Louis, MO; 1:100). Secondary antibody conjugated to Rhodamine-RedX (Jackson Laboratories, Suffolk, UK) was chosen as appropriate and used as recommended by the supplier. Where indicated, DNA was counterstained with 0.05 µg/ml 4′-6-diamidino-2-phenylindole (DAPI, Sigma, St Louis, MO). Coverslips were sealed in antifade solution (Vector, Burlingame, CA). Preparations were examined under an Olympus AX70 microscope using a 100×/1.35NA objective. Images were acquired using a SPOT charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI) controlled by ISO 2000 software (DeltaSistemi, Verona, Italy).

Statistical analysis

All data were obtained through at least three independent experiments and expressed as means ± standard deviations. We applied the unpaired *t*-test with Welch correction in order to assess the significance of RSV treatments effects. Values of $P < 0.05$ were considered statistically significant. Statistical analysis of data was performed using Graph Pad software Instat version 3.02 (Graph Pad Software, San Diego, CA).

Results

Induction of polyploidy, DNA damage and chromosome lost

The effects on chromosome segregation and micronuclei induction exerted by RSV are shown in Figures 1 and 2 and Table I. Figure 1 shows a CHO metaphase cell with diplochromosomes made up of four chromatids (a), a polyploid metaphase (b) and a binucleated cell with a micronucleus (c), as a consequence of RSV treatment. In Figure 2, the induction of both POL and ENDO cells after the treatment with RSV are shown. The increase became significant at doses of 80 and 120 µM.

As far as micronuclei induction, it followed a dose-dependent trend with a significant increase after 80 and 120 µM treatment (Table I). The NDI decreased with increasing dose being always significantly lower in treated versus control samples. In order to determine if the MN induction by RSV could be antagonised by concomitant treatment with a topoisomerase

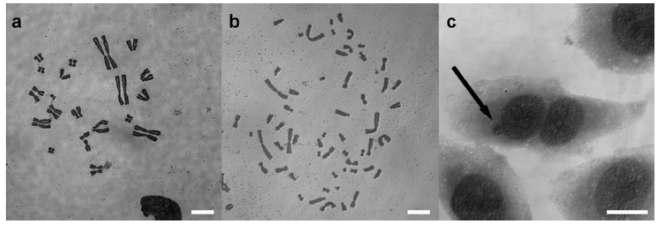

Fig. 1. ENDO cell with diplochromosomes (a) POL cell (b) and binucleated cell with one micronucleus (arrow) (c). Scale bar: 10 µM.

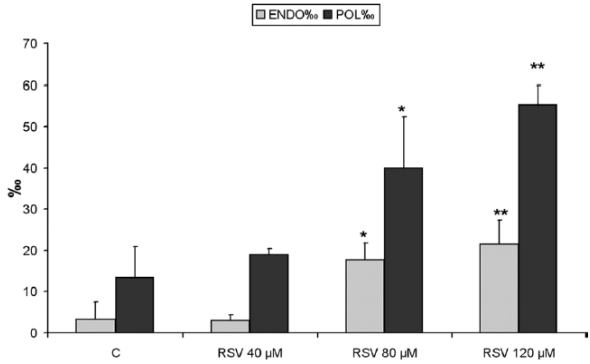

Fig. 2. Induction of POL and ENDO in CHO-K1 cells after RSV treatment lasting 6 h followed by 22 h of recovery time.

inhibitor, we performed combined treatments RSV + EtBr. As shown in Table I, we found a significant decrease of MN when the two drugs were provided simultaneously in comparison with single RSV treatment. In particular, the MN induced by 80 and 120 µM RSV were significantly reduced ($P < 0.01$ and $P < 0.05$, respectively) when EtBr concomitant treatment was performed. Furthermore, the loss of a whole chromosome as the consequence of an improper chromosome segregation induced by RSV treatment was analysed through CREST staining of MN. We found a significant increase of MN with centromere (CREST$^+$) after 2 h of treatment with 80 and 120 µM RSV (Table I).

Effects on mitotic progression

In order to check if a defective chromosome segregation could affect mitosis progression, we monitored the capacity of RSV treatment to modify the distribution of cells in the different phases of mitosis. In Figure 3, we show that RSV treatment caused an increase in the prometa/metaphase percentage with a concomitant decrease in the other mitotic stages. This effect is already evident at 80 and 120 µM RSV after 1 h of treatment; the increase of metaphases and the reduction of both prophases and ana-telophases reached the significance ($P < 0.01$ and $P < 0.05$, respectively) just after 120 µM dose. After 3 h of treatment all RSV doses induced a significant increase in prometa/metaphase percentage ($P < 0.01$). These results demonstrate that RSV-treated cells spend longer time in prometa/metaphase, possibly by trying to decatenate replicated DNA threads.

An incomplete decatenation of sister chromatids during mitosis can originate anaphases showing DNA bridges. In Figure 4 (upper part) is reported an immunofluorescence image showing the presence of DNA bridges in anaphase and ana-telophase. Immunostaining, with CREST, anti-tubulin antibodies and the DNA dye DAPI, demonstrates that DNA threads remain connecting the two groups of migrated chromosomes, as visualised by the CREST signals at the two poles. In the lower part of Figure 3, the frequency of anaphase bridges after RSV treatments is reported. Although the frequency of anaphase bridges is low (the highest value is 15%), the effect is evident at the highest dose (120 µM) both after 1 and 3 h treatment, reaching a significant value just after the longer treatment period ($P < 0.05$).

Discussion

In last decades, the natural compound RSV excited a great interest for its chemopreventive activity and beneficial effects

Table I. Micronuclei (MN‰) induced by RSV in CHO-K1 cells

	RSV treatment: 6 h			RSV treatment: 2 h	
	Recovering: 22 h cytochalasin B			Recovering: 4 h cytochalasin B	
	–EtBr	+EtBr	NDI	CREST$^-$	CREST$^+$
C	18.6 (11.1)	17.0 (2)	1.9 (0.04)	15.8 (3.3)	5.9 (1.8)
RSV 40 µM	32.1 (11.4)	22.3 (7)	1.76 (0.04)*	47.5 (18.1)	28.6 (8.9)*
RSV 80 µM	57.3 (13.5)**	34 (5.3)††	1.58 (0.04)**	56.3 (17.6)*	51.8 (12.6)**
RSV 120 µM	97.8 (19.9)**	63.7 (15)†	1.47 (0.07)**	66.6 (22.5)*	39.5 (3.4)**

The inhibitory action of EtBr on the clastogenic effect of RSV is shown when the two drugs are provided at the same time. The CREST staining highlighted the aneugenic effect of RSV. The NDI is reported in order to show the delayed proliferation after treatment with RSV. Values are mean with SD in bracket.
–EtBr: cells treated with RSV; **+EtBr**, cells treated with RSV and EtBr at the same time; **CREST$^-$**: MNlacking centromere; **CREST$^+$**: MN containing centromere.
*$P < 0.05$, **$P < 0.01$ performed by *t*-test treated cells versus control ones.
†$P < 0.05$, ††$P < 0.01$ performed by *t*-test +EtBr versus –EtBr.

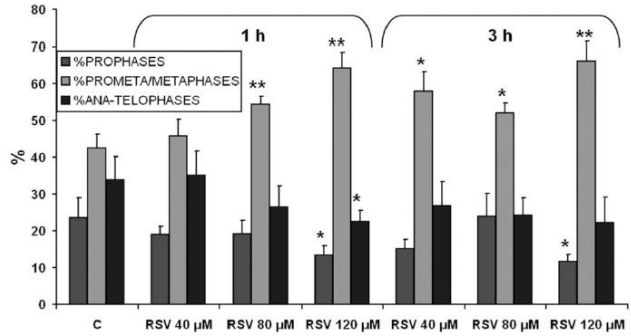

Fig. 3. Distribution of mitotic figures in CHO-K1 cells after RSV treatment lasting 1 and 3 h. $*P < 0.05$, and $**P < 0.01$ (unpaired *t*-test) when comparing treated samples with control ones.

against various diseases. There is a great amount of data collected both *in vitro* and *in vivo* showing that RSV is able to modulate various cell pathways in different cells. In particular as far as the induction of DNA damage, the data present in literature seem to converge on the claim that RSV could act on proliferating cells, principally cancer cells, inducing the expression of markers of DNA damage, such as γ-H2AX. DNA damage is one of the targets of chemoprevention and a

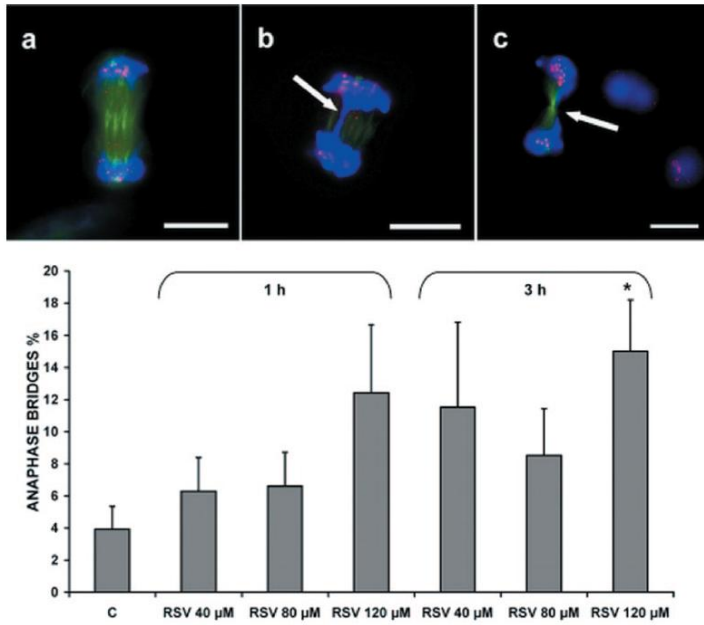

Fig. 4. Upper part: Immunofluorescence detection of anaphase bridges. Normal anaphase with a correct segregation of chromatids (**a**). Anaphase with a DNA bridge (**b**). Ana-telophase with a DNA bridge (**c**). The red signals represent kinetochores, the blue ones represent DNA and the green ones represent α tubulin. Scale bar: 5 µM. Lower part: percentage of anaphase bridges in CHO-K1 cells after treatment with different doses of RSV. $*P < 0.05$ at unpaired *t*-test.

desirable outcome is the induction of apoptosis and/or the arrest of cell proliferation. Thus, the ability of RSV in inducing DNA damage might also explain the activation of the checkpoints responsible for cell cycle arrest and, in some cases, apoptosis (4). A possible explanation for this behaviour could be also the inhibition of topoisomerases activity exerted by RSV. This will combine the delay in the S-phase progression with the increase in DNA damage. The results from our group showing the increase in γ-H2AX after RSV treatment in glioma cells associated with a delay in the S phase of the cell cycle are consistent with this hypothesis (11).

On the other hand, controversial data are present in literature about the influence of RSV treatment on the redox status of the cells. In fact, although its anti-oxidant power in certain conditions and concentrations is known, an enhancing number of data are now present in literature dealing with its pro-oxidant ability exerted in particular condition (for a review see (24)).

Therefore, it is conceivable that RSV can exert some genotoxic action mainly in actively proliferating cells that express high levels of TOPO2. In this study, we investigated the consequences of TOPO2 inhibition by RSV not only as micronuclei induction but also in respect to DNA decatenation and chromosome segregation, utilising the non-cancer cell line CHO-K1. CHO cells are established cells routinely employed in cytogenetic experiments with a substantially stable karyotype and a low spontaneous background of micronucleated cells (25). In this respect they can be a good surrogate of normal cells.

TOPO2 is an essential mammalian enzyme that topologically modifies DNA through the production of transient DSBs and is required for chromosome segregation during mitosis. TOPO2 is the target of widely used anti-cancer agents such as etoposide, anthracyclines (doxorubicin and daunorubicin) and mitoxantrone. These agents act by trapping DNA and TOPO2 in the so called 'cleavable complex' (26). Previous researches also suggest that inhibition of TOPO2 decatenatory activity triggers a G_2 checkpoint response, which delays the entry into mitosis due to insufficient decatenation of sister chromatids (27–29).

In this study, we show that RSV treatment induces micronuclei at the two highest doses as previously shown in human tumour cells (11). Indeed, one of the consequence of TOPO2 poisoning is the induction of DSBs that may produce MN, whereas the catalytic inhibition does not, except for some evidences reporting that treatment with ICRF-193 induced G_2 arrest and DNA damage (30). This statement is strongly supported by the reduction in micronucleated cells we obtained when combined treatments (RSV plus ethidium bromide) were performed. In fact catalytic inhibitors and intercalating agents, such as ethidium bromide, prevent the formation of TOPO2-induced DNA DSBs (31). We found a dose-dependent significant increase of micronuclei concomitant with a NDI decreases, indicating that the induction of DNA damage goes with a reduction of cell proliferation. This last result is in agreement with what is widely known on the ability of RSV to delay cell cycle progression especially in cancer cells, thus leading cells to death (8,32)

In this study, we also report that RSV causes an increase in polyploidy and endoreduplication, two phenomenon that occur when DNA replicates without a cell division (33). In fact endoreduplication is a different manifestation of polyploidy, which has to be considered as a proof of the prevention of decatenation of fully replicated chromosomes following TOPO2 inhibition. This event will lead to the failure of correct segregation at mitosis (34). Data are present in literature about the ability of different flavonoids to induce endoreduplication in CHO cells (35,36) at doses very close to those used in this article and these compounds are all able to interfere with TOPO2 activity.

The analysis of mitotic figures after treatment with RSV highlights a delay of mitotic progression with an accumulation of cells in the prometa/metaphase stage. This is in agreement with a reduction of TOPO2 activity respect to the resolution of intertwined chromatids, that is a critical step of the transition from metaphase to anaphase (15).

The outcome of a defective DNA decatenation is shown by the presence of DNA bridges during the ana-telophase stage, that could evolve in chromosome breaks. We found an increase of DNA bridges in the ana-telophase after treatment with RSV.

TOPO2 is an enzyme active in proliferating cells that through the same mechanism of action is involved in DNA synthesis and catenated DNA resolution and its action is performed during a large part of the cell cycle, from the S phase to the G_2/M phases. Our data show that RSV can affect TOPO2 activity at different stages of the cell cycle, during the DNA replication (through the induction of DSBs) and during the DNA segregation (through the induction of ENDO and POL cells). In this article, this 2-fold effect has been demonstrated through the presence of micronuclei $CREST^-$ that represent acentric chromosome fragments originated by DSBs and micronuclei $CREST^+$ that represent whole chromosomes originated by the failure of chromatid segregation.

On the whole, these results reinforce the statement of the role of RSV as a TOPO-poison and strongly suggest to explore more thoroughly its involvement in new therapeutic strategies.

References

1. Baur, J. A. and Sinclair, D. A. (2006) Therapeutic potential of resveratrol: the in vivo evidence. *Nat. Rev. Drug Discov.*, **5**, 493–506.
2. Jang, M., Cai, L., Udeani, G. O., *et al.* (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, **275**, 218–220.
3. Yu, W., Fu, Y.C. and Wang, W. (2011) Cellular and molecular effects of resveratrol in health and disease. *J. Cell. Biochem.*, **113**, 752–759.
4. Athar, M., Back, J. H., Kopelovich, L., Bickers, D. R. and Kim, A. L. (2009) Multiple molecular targets of resveratrol: anti-carcinogenic mechanisms. *Arch. Biochem. Biophys.*, **486**, 95–102.
5. Gatz, S. A., Keimling, M., Baumann, C., Dörk, T., Debatin, K. M., Fulda, S. and Wiesmüller, L. (2008) Resveratrol modulates DNA double-strand break repair pathways in an ATM/ATR-p53- and -Nbs1-dependent manner. *Carcinogenesis*, **29**, 519–527.
6. Denissova, N. G., Nasello, C. M., Yeung, P. L., Tischfield, J. A. and Brenneman, M. A. (2012) Resveratrol protects mouse embryonic stem cells from ionizing radiation by accelerating recovery from DNA strand breakage. *Carcinogenesis*, **33**, 149–155.
7. Smoliga, J. M., Baur, J. A. and Hausenblas, H. A. (2011) Resveratrol and health–a comprehensive review of human clinical trials. *Mol. Nutr. Food Res.*, **55**, 1129–1141.
8. Leone, S., Cornetta, T., Basso, E. and Cozzi, R. (2010) Resveratrol induces DNA double-strand breaks through human topoisomerase II interaction. *Cancer Lett.*, **295**, 167–172.
9. Bekker-Jensen, S. and Mailand, N. (2010) Assembly and function of DNA double-strand break repair foci in mammalian cells. *DNA Repair. (Amst)*, **9**, 1219–1228.
10. Jo, J. Y., Gonzalez de Mejia, E. and Lila, M. A. (2006) Catalytic inhibition of human DNA topoisomerase II by interactions of grape cell culture polyphenols. *J. Agric. Food Chem.*, **54**, 2083–2087.
11. Leone, S., Basso, E., Polticelli, F. and Cozzi, R. (2012) Resveratrol acts as a topoisomerase II poison in human glioma cells. *Int. J. Cancer*, **131**, E173–E178.

12. Villman, K., Ståhl, E., Liljegren, G., Tidefelt, U. and Karlsson, M. G. (2002) Topoisomerase II-alpha expression in different cell cycle phases in fresh human breast carcinomas. *Mod. Pathol.*, **15**, 486–491.
13. Cortés, F., Pastor, N., Mateos, S. and Domínguez, I. (2003) Roles of DNA topoisomerases in chromosome segregation and mitosis. *Mutat. Res.*, **543**, 59–66.
14. Coelho, P. A., Queiroz-Machado, J., Carmo, A. M., Moutinho-Pereira, S., Maiato, H. and Sunkel, C. E. (2008) Dual role of topoisomerase II in centromere resolution and aurora B activity. *PLoS Biol.*, **6**, e207.
15. Wang, L. H., Schwarzbraun, T., Speicher, M. R. and Nigg, E. A. (2008) Persistence of DNA threads in human anaphase cells suggests late completion of sister chromatid decatenation. *Chromosoma*, **117**, 123–135.
16. Burden, D. A. and Osheroff, N. (1998) Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochim. Biophys. Acta*, **1400**, 139–154.
17. McClendon, A. K. and Osheroff, N. (2007) DNA topoisomerase II, genotoxicity, and cancer. *Mutat. Res.*, **623**, 83–97.
18. Larsen, A. K., Escargueil, A. E. and Skladanowski, A. (2003) Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol. Ther.*, **99**, 167–181.
19. Deming, P. B., Cistulli, C. A., Zhao, H., Graves, P. R., Piwnica-Worms, H., Paules, R. S., Downes, C. S. and Kaufmann, W. K. (2001) The human decatenation checkpoint. *Proc. Natl Acad. Sci. USA*, **98**, 12044–12049.
20. Fenech, M., Kirsch-Volders, M., Natarajan, A. T., *et al.* (2011) Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*, **26**, 125–132.
21. Snyder, R. D. (2000) Use of catalytic topoisomerase II inhibitors to probe mechanisms of chemical-induced clastogenicity in Chinese hamster V79 cells. *Environ. Mol. Mutagen.*, **35**, 13–21.
22. Cortés, F. and Pastor, N. (2003) Induction of endoreduplication by topoisomerase II catalytic inhibitors. *Mutagenesis*, **18**, 105–112.
23. Kaufmann, W. K. (2009) Analysis of the topoisomerase II-dependent decatenation G2 checkpoint and checkpoint kinases in human cells. *Methods Mol. Biol.*, **582**, 155–166.
24. Basso, E., Leone, S. and Cozzi, R. (2012) Role of DNA damage in the antitumoral activity of resveratrol. In Delmas, D. (ed.), *Resveratrol: Source, Production and Health Benefits*. Nova Science Publishers, New York.
25. Aardema, M. J., Snyder, R. D., Spicer, C., *et al.* (2006) SFTG international collaborative study on in vitro micronucleus test III. Using CHO cells. *Mutat. Res.*, **607**, 61–87.
26. Pommier, Y., Leo, E., Zhang, H. and Marchand, C. (2010) DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem. Biol.*, **17**, 421–433.
27. Larsen, A. K., Skladanowski, A. and Bojanowski, K. (1996) The roles of DNA topoisomerase II during the cell cycle. *Prog. Cell Cycle Res.*, **2**, 229–239.
28. Wang, J. C. (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat. Rev. Mol. Cell Biol.*, **3**, 430–440.
29. Luo, K., Yuan, J., Chen, J. and Lou, Z. (2009) Topoisomerase IIalpha controls the decatenation checkpoint. *Nat. Cell Biol.*, **11**, 204–210.
30. Park, I. and Avraham, H. K. (2006) Cell cycle-dependent DNA damage signaling induced by ICRF-193 involves ATM, ATR, CHK2, and BRCA1. *Exp. Cell Res.*, **312**, 1996–2008.
31. Snyder, R. D. and Arnone, M. R. (2002) Putative identification of functional interactions between DNA intercalating agents and topoisomerase II using the V79 in vitro micronucleus assay. *Mutat. Res.*, **503**, 21–35.
32. Delmas, D., Solary, E. and Latruffe, N. (2011) Resveratrol, a phytochemical inducer of multiple cell death pathways: apoptosis, autophagy and mitotic catastrophe. *Curr. Med. Chem.*, **18**, 1100–1121.
33. Pastor, N., Cantero, G., Campanella, C. and Cortés, F. (2005) Endoreduplication induced in cultured Chinese hamster cells by different anti-topoisomerase II chemicals. Evidence for the essential contribution of the enzyme to chromosome segregation. *Mutat. Res.*, **582**, 11–19.
34. Cortés, F., Mateos, S., Pastor, N. and Domínguez, I. (2004) Toward a comprehensive model for induced endoreduplication. *Life Sci.*, **76**, 121–135.
35. Cantero, G., Campanella, C., Mateos, S. and Cortés, F. (2006) Topoisomerase II inhibition and high yield of endoreduplication induced by the flavonoids luteolin and quercetin. *Mutagenesis*, **21**, 321–325.
36. Neukam, K., Pastor, N. and Cortés, F. (2008) Tea flavanols inhibit cell growth and DNA topoisomerase II activity and induce endoreduplication in cultured Chinese hamster cells. *Mutat. Res.*, **654**, 8–12.

4 DISCUSSION

A great interest has emerged during last years around the use of natural compounds as a supporting remedy to medicine, in order to prevent the onset of human illnesses or to act as an adjuvant of conventional therapies. Herbs and plants are a natural source of bioactive compounds, namely phytochemicals, that are non-nutrient components of food exerting several beneficial effects for human health, in particular concerning the cure of cancer.

The intense research in the understanding of the way of action of several chemopreventive agents represents a big challenge because could provide the "magic bullet" in the cancer fight. Indeed phytochemicals are normal component of the diet, so they are not expensive, are present at low concentrations and for this reason they are considered safe [1]. Unfortunately the most of *in vitro* and *in vivo* studies concerning phytochemicals have been performed at concentrations that unlikely are reached in physiological conditions after a dietary intake. This represents a problem that must be considered for next investigations [2].

Starting from 1997 a growing amount of data, both *in vitro* and *in vivo*, have been produced about Resveratrol (RESV) showing its promising capacity in the treatment of cancer and other pathologies. At the moment around 60 clinical trials concerning the assessment of safety of RESV and for a potential use as remedy for various illnesses are in course (*www.clinicaltrials.gov*) [3]. Moreover RESV is a compound that acts on many factors and biochemical pathways and this represents a complication because it becomes difficult to establish a pattern of action [4].

Here, starting from the study of the antiproliferative role of RESV in cancer cells, we have arrived to conclude that its chemopreventive/chemotherapeutic action can be in part explained by the interaction with TOPO2.

Our first observation, through a citofluorimetric bi-parametric assay, concerning a delay in the S-phase of cell cycle accompanied by the maximal induction of DNA DSBs, in terms of phosphorylation of the histone H2AX at this stage in glioma cells, confirmed the extended literature about the anti-proliferative role of the stilbene in cancer cells. On the other hand we showed also that RESV was able to induce DNA DSBs, an aspect poor investigated until now.

Many evidences indicate that RESV is able to arrest cell proliferation at various stages of cell cycle. The stop in the G1-phase can occur through the

p53 dependant activation of p21. p21 in turn reduces the activity of the cyclinD/cdk4 complex. The inhibition of the binding of transcription factors (AP1 and NFκB) exerted by RESV causes a decrease in protein expression of cyclin D1, D2, E and cdk4, 6, 2. The G1/S transition is blocked by a reduced hyperphosphorylation of retinoblastoma protein-tumor suppressor (pRb) [5].
The S-phase arrest is mediated by RESV through the disruption of the dephosphorylation process of cdk1 that is a key regulator of the progression in the S-phase. Indeed it increases cyclin A and B expression leading in this manner to an accumulation of inactive cdk1 [6]. Moreover RESV can block cells at this stage acting directly on DNA synthesis, through the inhibition of the ribonucleotide reductase activity and DNA polymerase [7].
The inactivation of cdk1 is also responsible of the accumulation of cells in the G2-phase. The increase of cyclin A and B exerted by RESV causes a block in G2/M [5].
Histone H2AX, a variant of H2A, is phosphorylated at its carboxy-terminal Serine 139 as one of the earliest cellular response to the induction of DNA DSBs [8]. Once phosphorylated, γ-H2AX foci rapidly form over chromatin regions on either side of a DSB facilitating repair through relocalization and focusing factors to the site of breaks. γ-H2AX also functions in the end-joining of DSBs by serving as an anchor for holding broken chromosomal ends in close proximity [9].
So both our results, namely a delay in the S-phase with a concomitant induction of DNA DSBs, have let us to suppose a putative interaction of RESV with TOPO2. We demonstrate here that RESV is able to inhibit the decatenation activity of TOPO2 through an *in vitro* test, adding a new interesting element for the explanation of its chemopreventive activity. This result is in agreement with what previously showed by Cho et al. who analysed a great number of natural compounds including RESV tested for their TOPO enzymes inhibitory activity [10]. Jo et al. [11] showed that polyphenolic fractions from grape cell culture were potent inhibitor of human TOPO2, utilizing the same assay. Similar results were also achieved by Yamada et al. [12] studying four new RSV oligomers.

TOPO2 is an enzyme mainly expressed in proliferating cells from the S-phase, where it is involved in the resolution of DNA supercoiled, to the G2/M phase, where it is involved in the decatenation of sister chromatids driving in this manner a proper chromosome segregation. These evidences make TOPO2 a preferential target of cancer chemotherapy. In fact,many anticancer drugs have been designed in order to inhibit TOPO2 activity. There are two principal classes of TOPO2 inhibitors: catalytic inhibitors,

which act by blocking the catalytic cycle at various stages without inducing any DNA damage and TOPO2 poisons, which stabilize the covalent complex DNA-TOPO2, namely the "cleavage complex", provoking in this manner DNA DSBs [13].

Utilizing different approaches we demonstrated for the first time that RESV acts as a TOPO2 poison in glioma cells.
An *in silico* docking simulation provided us a first evidence about a putative stabilization of the covalent complex DNA-TOPO2 exerted by RESV. Then through the *In Vivo* Complex of Enxyme Assay (ICE) we showed as the stilbene is able to trap both TOPO2 and DNA in a non-covalent manner forming the cleavage complex. The ICE Assay represents a validate test hugely utilised to assess new chemical entities for their ability to inhibit DNA topoisomerases, in particular to examine the topoisomerase covalent complexes *in vivo* formation [14].
Since one of the consequences of TOPO2 poisoning is the induction of DNA DSBs, we analysed the presence of DNA damage through the Cytokinesis-Block Micronucleus Assay (CBMN) and the activation of DNA damage signalling pathway. Our data showed a significant increase of micronuclei and the activation of the ATM/ATR signalling pathway in glioma cells. The induction of DSBs through TOPO2 poisoning by RESV treatment is also confirmed by γ-H2AX expression immediately after treatment and by its persistence after a recovery period. These data together with the increase in the phosphorylated form of ATM and Chk2 expression lead us to conclude that RESV induced DNA damage is sensed by early signal transducers that activate S phase arrest.
Tyagi and co-workers [15] demonstrated that RESV induces S phase arrest via Tyr15 phosphorylation of Cdc2 in human ovarian carcinoma cells. Further they detected the activation of checkpoint kinases Chk1 and Chk2, which in turn were activated via ATM /ATR kinase in response to DNA damage. In the same study they observed as RESV also increased γ-H2AX, which is known to be phosphorylated by ATM/ATR in response to DNA damage. The involvement of these molecules in RESV-induced S-phase delay was also supported by the data showing that the addition of ATM/ATR inhibitor caffeine reverses RESV-caused activation of ATM/ATR–Chk1/2 as well as the phosphorylation of Cdc25C, Cdc2, γ-H2AX, and S phase arrest. On the contrary RESV showed only marginal S phase arrest in normal human foreskin fibroblasts with undetectable level of γ-H2AX [15].
Studying the effects of RESV on genome stability, Gatz and co-workers showed that it inhibits both homologous recombination (HR) and non-

homologous end joining (NHEJ) [16]. They observed an ATM–p53-dependent pathway of HR inhibition exerted by RESV, providing also evidences for an ATM/ATR–Nbs1-dependent inhibition of NHEJ after RESV treatment. These data highlighted as the activation of ATM and/or ATR carries on a central role in the effects mediated by RESV respect to DNA integrity.

During last years evidences are emerging indicating that several phytochemicals with chemopreventive activity can interfere on topoisomerases catalytic cycle. Ellagic acid it has been described as an inhibitor of TOPO2 by competing with ATP for the binding to the enzyme. The effect has been detected both with docking simulation and decatenation test [17]. Chlorogenic acid, a coffee constituent, is able to stabilize complexes between DNA and both TOPO1 and TOPO2 and as a consequence it induces DNA damage measured through Comet Assay and H2AX phosphorylation [18]. Other bioflavonoids such as genistein, luteolin and quercetin have been previously described as TOPO2 poisons [19]. Tea flavanols, namely epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), catechin (CA) and epicatechin (EC), act as catalytic inhibitors of TOPO2 activity in hamster ovary AA8 cells. In the range of concentrations that caused TOPO2 inhibition, they induced a high level of endoreduplication [20]. There are also evidences for a TOPO2 poisoning activity of EGCG [21]. Curcumin, the major active component of the spice turmeric, acts as an anticancer agent by inducing apoptosis in cancer cells. It has been hypothesised that the DNA damage induced by TOPO2 poisoning is a possible mechanism by which curcumin initiates apoptosis [22].

In the last part we performed a mechanistic study regarding the interaction between RESV and TOPO2 in CHO cells, that are non-cancer proliferating cells. In particular we analysed whether RESV activity could affect the chromosome stability. In regard to this aspect CHO cells represent an ideal experimental model because they have a mammalian origin and their cell cycle consists of 15-16 hours. Moreover they own a modal number of chromosomes rounds up 21, so facilitating the performing of cytogenetic analysis.
TOPO2 is directly involved in the human decatenation checkpoint that occurs at the G2/M stage of cell cycle. In this phase chromatid catenation is actively monitored in human cells, with progression from G2 to mitosis being inhibited when chromatids are insufficiently decatenated. TOPO2 catalytic inhibitors prevent chromatid decatenation, then produce mitotic delay without producing DNA strand breaks [23,24]. Our evidences showed

as the presence of RESV in CHO cells can affect all these aspect of TOPO2 activity.
We found an increase of micronuclei (as we did in glioma cells), since one of the consequence of TOPO2 poisoning is the induction of DSBs that may produce MN; on the contrary, the catalytic inhibition does not, except for some evidences reporting that treatment with ICRF-193 induced G2 arrest and DNA damage [25].
Our results have been strongly supported by the reduction in micronucleated cells obtained when we performed combined treatments (RESV plus EtBr). In fact catalytic inhibitors and intercalating agents, such as EtBr, prevent the formation of TOPO2-induced DNA DSBs [26]. We found a dose-dependent significant increase of micronuclei concomitant with a NDI decreases, indicating that the induction of DNA damage goes with a reduction of cell proliferation.
We also reported that RESV causes an increase in polyploidy and endoreduplication, two phenomena that occur when DNA replicates without a cell division [27]. In fact endoreduplication is a different manifestation of polyploidy, which has to be considered as a proof of the prevention of decatenation of fully replicated chromosomes following TOPO2 inhibition. This event will lead to the failure of correct segregation at mitosis [28]. Similarly Cantero and co-workers demonstrated that flavonoids, luteolin and quercetin are TOPO2 inhibitors by conducting a comparative study of their effect on TOPO2 activity in Chinese hamster ovary AA8 cells. Concentrations of luteolin and quercetin that inhibited TOPO2 catalytic activity resulted in extraordinarily high yields of metaphases showing diplochromosomes [29].
The analysis of mitotic figures after treatment with RESV highlights a delay of mitotic progression with an accumulation of cells in the prometa/metaphase stage. This is in agreement with a reduction of TOPO2 activity respect to the resolution of intertwined chromatids, that is a critical step of the transition from metaphase to anaphase [30]. The outcome of a defective DNA decatenation is shown by the presence of DNA bridges during the ana-telophase stage, that could evolve in chromosome breaks. We found an increase of DNA bridges in the ana-telophase after treatment with RESV.

On the whole the data obtained have confirmed the chemopreventive role of RESV in cancer cells and on the other hand have emphasized a new aspect of this compound that has been little investigated in the past. Indeed we showed here for the first time that RESV is able to poisoning TOPO2, inducing in this manner DNA DSBs and activating pathways involved in

DNA damage signalling and cell cycle control in cancer cells. Moreover it affects chromosome stability at various levels in CHO cells as a consequence of TOPO2 poisoning. These evidences create a new insight into RESV landscape, supporting the idea that it could be utilised as chemopreventive in cancer prevention and/or as an adjuvant of conventional therapies (Fig. 7).

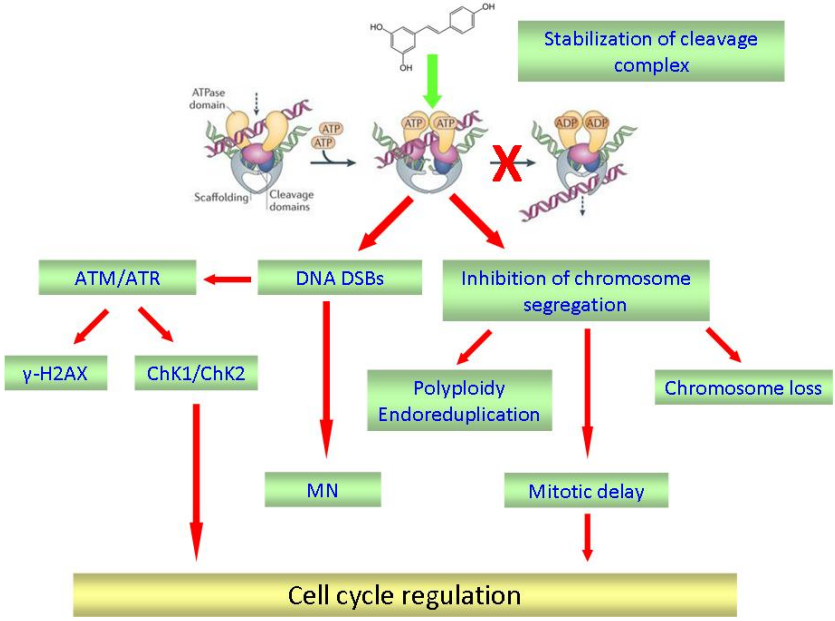

Figure 7. Summary of the effects obtained after the stabilization of the cleavage complex exerted by RESV. On one hand it induces DNA damage, leading to the production of micronuclei (MN) and to the activation of the ATM/ATR signalling pathway. On the other hand it affects chromosome segregation, provoking chromosome loss, polyploidy, endoreduplication and mitotic delay. Both these effects contribute to the delay in cell cycle progression.

4.1 REFERENCES

1. Gullett, N.P., Ruhul Amin, A.R., Bayraktar, S., Pezzuto, J.M., Shin, D.M., Khuri, F.R., Aggarwal, B.B., Surh, Y.J. and Kucuk, O. (2010) Cancer prevention with natural compounds. *Semin Oncol*, **37**, 258-81.
2. Scott, E., Steward, W.P., Gescher, A.J. and Brown, K. (2012) Resveratrol in human cancer chemoprevention - Choosing the 'right' dose. *Mol Nutr Food Res*, **56**, 7-13.
3. Smoliga, J.M., Baur, J.A. and Hausenblas, H.A. (2011) Resveratrol and health - A comprehensive review of human clinical trials. *Mol Nutr Food Res*, **55**, 1129-41.
4. Whitlock, N.C. and Baek, S.J. (2012) The Anticancer Effects of Resveratrol: Modulation of Transcription Factors. *Nutr Cancer*.
5. Kraft, T.E., Parisotto, D., Schempp, C. and Efferth, T. (2009) Fighting cancer with red wine? Molecular mechanisms of resveratrol. *Crit Rev Food Sci Nutr*, **49**, 782-99.
6. Delmas, D., Passilly-Degrace, P., Jannin, B., Cherkaoui Malki, M. and Latruffe, N. (2002) Resveratrol, a chemopreventive agent, disrupts the cell cycle control of human SW480 colorectal tumor cells. *Int J Mol Med*, **10**, 193-9.
7. Stivala, L.A., Savio, M., Carafoli, F., Perucca, P., Bianchi, L., Maga, G., Forti, L., Pagnoni, U.M., Albini, A., Prosperi, E. and Vannini, V. (2001) Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. *J Biol Chem*, **276**, 22586-94.
8. Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. and Bonner, W.M. (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem*, **273**, 5858-68.
9. Bassing, C.H. and Alt, F.W. (2004) H2AX may function as an anchor to hold broken chromosomal DNA ends in close proximity. *Cell Cycle*, **3**, 149-53.
10. Cho, K.H., Pezzuto, J.M., Bolton, J.L., Steele, V.E., Kelloff, G.J., Lee, S.K. and Constantinou, A. (2000) Selection of cancer chemopreventive agents based on inhibition of topoisomerase. *Eur J Cancer*, **36**, 2146-56.

11. Jo, J.Y., Gonzalez de Mejia, E. and Lila, M.A. (2006) Catalytic inhibition of human DNA topoisomerase II by interactions of grape cell culture polyphenols. *J Agric Food Chem*, **54**, 2083-7.
12. Yamada, M., Hayashi, K., Hayashi, H., Tsuji, R., Kakumoto, K., Ikeda, S., Hoshino, T., Tsutsui, K., Ito, T., Iinuma, M. and Nozaki, H. (2006) Nepalensinols D-G, new resveratrol oligomers from Kobresia nepalensis (Cyperaceae) as potent inhibitors of DNA topoisomerase II. *Chem Pharm Bull (Tokyo)*, **54**, 354-8.
13. Larsen, A.K., Escargueil, A.E. and Skladanowski, A. (2003) Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol Ther*, **99**, 167-81.
14. Nitiss, J.L., Soans, E., Rogojina, A., Seth, A. and Mishina, M. (2012) Topoisomerase assays. *Curr Protoc Pharmacol*, **Chapter 3**, Unit 3 3.
15. Tyagi, A., Singh, R.P., Agarwal, C., Siriwardana, S., Sclafani, R.A. and Agarwal, R. (2005) Resveratrol causes Cdc2-tyr15 phosphorylation via ATM/ATR-Chk1/2-Cdc25C pathway as a central mechanism for S phase arrest in human ovarian carcinoma Ovcar-3 cells. *Carcinogenesis*, **26**, 1978-87.
16. Gatz, S.A., Keimling, M., Baumann, C., Dork, T., Debatin, K.M., Fulda, S. and Wiesmuller, L. (2008) Resveratrol modulates DNA double-strand break repair pathways in an ATM/ATR-p53- and -Nbs1-dependent manner. *Carcinogenesis*, **29**, 519-27.
17. Furlanetto, V., Zagotto, G., Pasquale, R., Moro, S. and Gatto, B. (2012) Ellagic acid and polyhydroxylated urolithins are potent catalytic inhibitors of human topoisomerase II: an in vitro study. *J Agric Food Chem*, **60**, 9162-70.
18. Burgos-Moron, E., Calderon-Montano, J.M., Orta, M.L., Pastor, N., Perez-Guerrero, C., Austin, C., Mateos, S. and Lopez-Lazaro, M. (2012) The Coffee Constituent Chlorogenic Acid Induces Cellular DNA Damage and Formation of Topoisomerase I- and II-DNA Complexes in Cells. *J Agric Food Chem*.
19. Bandele, O.J. and Osheroff, N. (2007) Bioflavonoids as poisons of human topoisomerase II alpha and II beta. *Biochemistry*, **46**, 6097-108.
20. Neukam, K., Pastor, N. and Cortes, F. (2008) Tea flavanols inhibit cell growth and DNA topoisomerase II activity and induce endoreduplication in cultured Chinese hamster cells. *Mutat Res*, **654**, 8-12.
21. Bandele, O.J., Clawson, S.J. and Osheroff, N. (2008) Dietary polyphenols as topoisomerase II poisons: B ring and C ring

substituents determine the mechanism of enzyme-mediated DNA cleavage enhancement. *Chem Res Toxicol*, **21**, 1253-60.
22. Martin-Cordero, C., Lopez-Lazaro, M., Galvez, M. and Ayuso, M.J. (2003) Curcumin as a DNA topoisomerase II poison. *J Enzyme Inhib Med Chem*, **18**, 505-9.
23. Luo, K., Yuan, J., Chen, J. and Lou, Z. (2009) Topoisomerase IIalpha controls the decatenation checkpoint. *Nat Cell Biol*, **11**, 204-10.
24. Deming, P.B., Cistulli, C.A., Zhao, H., Graves, P.R., Piwnica-Worms, H., Paules, R.S., Downes, C.S. and Kaufmann, W.K. (2001) The human decatenation checkpoint. *Proc Natl Acad Sci U S A*, **98**, 12044-9.
25. Park, I. and Avraham, H.K. (2006) Cell cycle-dependent DNA damage signaling induced by ICRF-193 involves ATM, ATR, CHK2, and BRCA1. *Exp Cell Res*, **312**, 1996-2008.
26. Snyder, R.D. and Arnone, M.R. (2002) Putative identification of functional interactions between DNA intercalating agents and topoisomerase II using the V79 in vitro micronucleus assay. *Mutat Res*, **503**, 21-35.
27. Pastor, N., Cantero, G., Campanella, C. and Cortes, F. (2005) Endoreduplication induced in cultured Chinese hamster cells by different anti-topoisomerase II chemicals. Evidence for the essential contribution of the enzyme to chromosome segregation. *Mutat Res*, **582**, 11-9.
28. Cortes, F., Mateos, S., Pastor, N. and Dominguez, I. (2004) Toward a comprehensive model for induced endoreduplication. *Life Sci*, **76**, 121-35.
29. Cantero, G., Campanella, C., Mateos, S. and Cortes, F. (2006) Topoisomerase II inhibition and high yield of endoreduplication induced by the flavonoids luteolin and quercetin. *Mutagenesis*, **21**, 321-5.
30. Wang, L.H., Schwarzbraun, T., Speicher, M.R. and Nigg, E.A. (2008) Persistence of DNA threads in human anaphase cells suggests late completion of sister chromatid decatenation. *Chromosoma*, **117**, 123-35.