Abstract. Resveratrol has been reported to inhibit or induce DNA damage, depending upon the type of cell and the experimental conditions. Dietary resveratrol is present in the body predominantly as metabolites and limited data is available concerning the activities of these metabolic products. In the present study, physiologically obtainable levels of the resveratrol metabolites resveratrol-3-O-glucuronide, resveratrol-4'-O-glucuronide and resveratrol-3-O-sulfate were evaluated for their ability to protect Jurkat T cells against DNA damage induced by the topoisomerase I inhibitors camptothecin and topotecan. The cells were pretreated for 24 h with 10 µM resveratrol aglycone or each resveratrol metabolite prior to the induction of DNA damage with camptothecin or topotecan. In separate experiments, the cells were co-treated with resveratrol or its metabolites, and a topoisomerase I inhibitor. The detection of histone 2AX phosphorylation and terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) were used to determine DNA damage, and apoptosis was measured using an antibody against cleaved poly ADP-ribose polymerase. It was identified that pretreatment of the cells with resveratrol-3-O-glucuronide and resveratrol-4'-O-glucuronide reduced the mean fluorescence intensity of staining for DNA strand breaks following treatment with camptothecin, while the percentage of cells undergoing apoptosis was unchanged. However, pretreatment of the cells with resveratrol aglycone increased the DNA damage and apoptosis induced by the drugs. These results suggest that the glucuronide metabolites of resveratrol partially protected the cells from DNA damage, but did not influence the induction of cell death by camptothecin and topotecan. These data suggest that resveratrol aglycone treatment may be beneficial for treating types of cancer that have direct contact with resveratrol prior to its metabolism, including gastrointestinal cancers, which are routinely treated with topoisomerase I inhibitors.

Introduction

Resveratrol, an anti-fungal phytochemical that occurs in grapes, blueberries, mulberries, cranberries and red wine, is rapidly metabolized by the intestine and liver following oral consumption (1–6). The major phase II metabolites of resveratrol include glucuronidated, sulfated and methylated products (2–6). Intestinal bacteria have been reported to break down resveratrol to metabolic products including benzoic, phenylacetic and propionic acids (7,8). Dihydroresveratrol, another metabolic product of gut bacteria, has been identified in the form of glucuronidated and sulfated products in plasma and urine following the consumption of resveratrol (9,10). Gut microbiota produce other conversion products of resveratrol subsequent to ingestion, including 3,4'-dihydroxy-trans-stilbene and 3,4'-dihydroxybibenzyl (11–13). A previous human study with oral doses of ≤5 g of resveratrol per day demonstrated that the peak plasma levels of resveratrol, resveratrol-3-O-glucuronide, resveratrol-4'-O-glucuronide and resveratrol-3-O-sulfate were 4.2, 17.1, 10.2 and 18.3 µM, respectively (2). These data indicate that high concentrations of resveratrol glucuronide and sulfate metabolites are achieved in the plasma following the dietary intake of resveratrol. Pharmacokinetic analysis of resveratrol and its metabolites in that study revealed that the phytochemical products remain in the plasma for 5–8 h (2).

Camptothecin is a topoisomerase I inhibitor that induces cytotoxicity by the generation of DNA strand breaks (14). Topotecan is a water-soluble derivative of camptothecin. Camptothecin compounds act primarily by binding to and stabilizing the topoisomerase I-DNA complex, which then collides with the replication fork during S phase of the cell cycle. This collision results in topoisomerase I-linked DNA breaks, the formation of double-strand DNA breaks and the irreversible arrest of DNA replication. Camptothecin compounds inhibit transcription by a similar mechanism: By...
binding to the topoisomerase I-DNA complex and colliding with the RNA polymerase complex, leading to the arrest of RNA synthesis and the generation of single-strand DNA breaks (14).

DNA strand breaks result in the activation of the DNA repair machinery and, if repair is not possible, apoptosis. DNA strand breaks can be quantified by several methods, including by the antibody-mediated detection of phosphorylated histone subunit 2AX (H2AX) and labeling the ends of broken strands with the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay (15,16). H2AX is a histone 2A isoform that is present at levels of 2-25% in the histone core of the DNA complex. It is phosphorylated, in response to DNA strand breaks, by the phosphatidylinositol-3 kinase-like family of kinases, including ataxia telangiectasia mutated (ATM) and ATM-and Rad3-related (ATR), and by DNA-dependent protein kinase (DNA-PK) (17,18).

Resveratrol aglycone has been studied extensively in vitro to determine its mechanisms of action; however, for these studies have utilized high concentrations of resveratrol aglycone (50-100 µM) that are not yet achievable in vivo, particularly in the plasma. Considering that resveratrol aglycone reached a peak concentration of 4.2 µM in the blood following the ingestion of 5 g per day (2), the data from the majority of in vitro studies are not likely to be reflective of the actual activity once resveratrol is absorbed and metabolized. There is also limited information on the activity of resveratrol metabolites.

In the present study, using Jurkat T cells as a model representing a cell type that occurs in the blood, camptothecin and topotecan were applied in order to determine whether or not glucuronidated and sulfated metabolites of resveratrol were able to increase or inhibit the DNA damage induced by these drugs. DNA damage was measured by determining the extent of H2AX phosphorylation and TUNEL staining. Apoptosis in the cells was determined by measuring the extent of the cleavage of poly ADP-ribose polymerase (PARP). The cells were pretreated with physiological levels of resveratrol-3-O-glucuronide, resveratrol-4’-O-glucuronide or resveratrol-3-O-sulfate prior to the induction of DNA damage by camptothecin and topotecan, or co-treated with metabolites and drugs. Flow cytometry was used to measure the extent of DNA damage and apoptosis, and the activities of the resveratrol metabolites were compared with equivalent amounts of the resveratrol aglycone.

Materials and methods

Cell culture and chemicals. Jurkat acute lymphoblastic T leukemia cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C with 5% CO₂ in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 50 IU/ml penicillin, 50 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 1 mM sodium pyruvate and 2 mM L-glutamine (Invitrogen; Thermo Fisher Scientific, Inc.). Trans-resveratrol (≥99% pure), dimethyl sulfoxide (DMSO; vehicle) and topotecan hydrochloride hydrate (≥98%) were purchased from Sigma-Aldrich (Merck KGaA). Trans-resveratrol 3-O-D-glucuronide (≥95%), trans-resveratrol 4’-O-D-glucuronide (≥95%) and resveratrol-3-O-sulfate (≥98%) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Camptothecin (≥98%) was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA).

H2AX phosphorylation and apoptosis measurements. Jurkat T cells were cultured at a concentration of 0.5x10⁶ cells/ml in 24-well plates (Sarstedt, Inc., Newton, NC, USA) with 0.1% DMSO (control) or 10 µM each of resveratrol aglycone, resveratrol-3-O-glucuronide, resveratrol-4’-O-glucuronide or resveratrol-3-O-sulfate for 24 h in the previously described conditions. Cells were then washed with RPMI-1640 and incubated with 5 µM camptothecin or 10 µM topotecan for a further 4 h in the same conditions. The cells were fixed and permeabilized for intracellular staining using the Fixation/Permeabilization Solution kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s protocol. For each treatment group, 10⁶ cells were stained with phycoerythrin-conjugated anti-cleaved PARP (0.06 µg/20 µl test; cat. no. 552933) and Alexa Fluor-conjugated anti-phosphorylated H2AX (0.125 µg/5 µl test; cat. no. 56-447) (both from BD Biosciences) antibodies. Following 30 min incubation on ice, the cells were fixed in 1% paraformaldehyde prepared in phosphate-buffered saline (Sigma-Aldrich; Merck KGaA). The cells were assessed with an LSRFortessa flow cytometer and the results analyzed using FACSDiva software v8.0.1 (both from BD Biosciences). A total of 30,000 events were collected per measurement, subsequent to gating to exclude debris.

H4A assay. Cells were pretreated with resveratrol and its metabolites for 24 h as previously described. Cells were then washed with RPMI medium and treated with 5 µM camptothecin or 10 µM topotecan for a further 4 h. In separate experiments, the cells were co-treated with resveratrol or its metabolites, plus one of the topoisomerase-inhibitor drugs at doses as previously described for a total of 4 h. DNA strand breaks were labeled with a TUNEL assay (Apo-Direct kit; BD Biosciences) with fluorescein isothiocyanate (FITC)-labelled dUTP according to the manufacturer’s protocol, with the following modifications: The fixation and membrane permeabilization step using 70% v/v ethanol was performed for >18 h at -20°C and the end-labeling reaction with the terminal transferase was 4 h at 37°C. Labeled cells were treated at room temperature for 30 min with RNase and propidium iodide (PI) as provided in the Apo-Direct kit at the concentrations recommended by the manufacturer. A total of 30,000 events were collected on the LSRFortessa flow cytometer for each treatment. The end-labeling of DNA strand breaks was evaluated, subsequent to gating singlet populations, based on the fluorescence of the PI area vs. width.

Statistical analysis. Statistical analyses were performed using GraphPad Prism v6.05 (GraphPad Software, Inc., La Jolla, CA, USA) and the data were presented as the mean ± standard error of the mean. P-values were obtained using one-way analysis of variance with Tukey’s multiple comparisons test to evaluate the significance of differences between the means of the treatment groups. P<0.05 was considered to indicate a statistically significant difference.
Results

Phosphorylation of H2AX. The histone protein H2AX is phosphorylated in response to DNA damage. The extent of phosphorylated H2AX was assessed by flow cytometry to determine the effects of resveratrol and its metabolites on DNA damage induced by camptothecin and topotecan. The cells were pretreated with 10 µM of resveratrol or its metabolites, for 24 h prior to a 4-h treatment with the DNA-damaging agents. Resveratrol aglycone was used at a concentration of 10 µM as a comparative control for DNA damage and apoptosis. The proportion of cells with phosphorylated H2AX and the mean fluorescence intensity (MFI) of this population were measured to determine alterations in DNA damage attributable to the phytochemicals. Treatment of cells with resveratrol aglycone alone for 24 h increased the percentage of cells with phosphorylated H2AX compared with untreated control cells (Fig. 1A; P<0.05). Additionally, resveratrol aglycone pretreatment with the subsequent addition of camptothecin or topotecan was associated with an increased percentage of cells with phosphorylated H2AX compared with cells treated only with the topoisomerase inhibitors (Fig. 1B and C; P<0.05), suggesting an additive effect. A corresponding increase in the MFI of H2AX staining was observed following the pretreatment of Jurkat cells with resveratrol aglycone followed by the DNA damaging agents (data not shown). In camptothecin-treated cells, resveratrol aglycone increased the MFI from 5,332±156 in the control group to 7,000±198; and in topotecan-treated cells, resveratrol aglycone increased the MFI from 5,105±115 (control) to 6,747±209 (P<0.05), indicating an increase in DNA damage. The resveratrol glucuronide and sulfate metabolites did not significantly alter the percentage of H2AX-positive cells or the MFI of H2AX staining following drug treatments.

DNA strand breaks following pretreatment of cells with phytochemicals. The TUNEL assay provides a direct measurement of DNA damage by labeling the DNA break-point ends. In cells treated with camptothecin or topotecan, only pretreatment with resveratrol aglycone resulted in a significant increase of the percentage of cells with detectable DNA breaks compared with the control group (Fig. 2; P<0.05), which confirms the result observed for H2AX phosphorylation. However, in camptothecin-treated cells, pretreatment with resveratrol-3-glucuronide or resveratrol-4'-glucuronide significantly reduced the MFI compared with the control group, suggesting that the extent of damage (or number of DNA strand breaks) per cell was attenuated by pretreatment with these metabolites (Fig. 3, P<0.05). In the topotecan-treated cells, there were no differences in MFI between any of the groups (data not shown).
Induction of apoptosis following pretreatment with resveratrol and metabolites. The topoisomerase I inhibitors camptothecin and topotecan function to produce DNA damage, cell cycle arrest and apoptosis. The extent of apoptosis was measured in the Jurkat cells using an antibody against the cleaved form of PARP, as cleavage of PARP occurs at a late stage in the apoptotic pathway. The cells were pretreated with resveratrol or its metabolites for 24 h, and DNA damage was induced by the topoisomerase I inhibitors. Cells treated only with resveratrol or its metabolites for 24 h did not induce PARP cleavage compared with untreated cells (Fig. 4A). A similar reduction in the percentage of cells with DNA damage was observed for cells co-treated with topotecan and resveratrol aglycone, although this reduction did not reach statistical significance. A reduction in DNA damage was observed between topotecan treatment alone (control) and co-treatment with topotecan plus resveratrol aglycone and its metabolites for 24 h without drugs. The topoisomerase I inhibitor-treated cells, only pretreatment with 10 µM resveratrol aglycone increased the percentage of cells with cleaved PARP compared with the cells treated only with resveratrol or its metabolites (Fig. 4B and C; P<0.05), whereas the resveratrol metabolites had no effect on the level of apoptosis induced by treatment with camptothecin and topotecan.

Co-treatment with resveratrol metabolites and topoisomerase I inhibitors. Jurkat cells were co-treated with resveratrol aglycone or its metabolites plus a topoisomerase I inhibitor for 4 h, and DNA damage was analyzed using the TUNEL assay. Treatment with only resveratrol or one of its metabolites for 4 h did not increase the extent of DNA strand breakage detected by the assay (Fig. 5A). In the topoisomerase I inhibitor-treated cells, only pretreatment with 10 µM resveratrol aglycone increased the percentage of cells with cleaved PARP compared with the cells treated only with the drugs (Fig. 4B and C; P<0.05), whereas the resveratrol metabolites had no effect on the level of apoptosis induced by treatment with camptothecin and topotecan.

Figure 3. Pretreatment with resveratrol-3-O-glucuronide and resveratrol-4’-O-glucuronide decreases the number of DNA strand breaks induced by camptothecin in Jurkat cells, as determined by MFI. The control group was pretreated with 0.1% dimethyl sulfoxide prior to camptothecin. *P<0.05 compared with control group. Data represent the MFI ± standard error of the mean from 4 separate experiments. MFI, mean fluorescence intensity; R, resveratrol aglycone; R3G, resveratrol-3-O-glucuronide; R4’G, resveratrol-4’-O-glucuronide; R3S, resveratrol-3-O-sulfate.

Figure 4. Pretreatment with resveratrol aglycone, but not its metabolites, increases the percentage of Jurkat cells with cleaved PARP following the induction of DNA damage by camptothecin or topotecan. (A) Percentage of cells with cleaved PARP following treatment with 10 µM resveratrol or its metabolites. The control group was treated with 0.1% dimethyl sulfoxide (vehicle). (B) Percentage of cells with cleaved PARP following pretreatment with 10 µM resveratrol or metabolites prior to treatment with 5 µM camptothecin. The control group was cells pretreated with vehicle prior to camptothecin. (C) Percentage of cells with cleaved PARP following pretreatment with 10 µM resveratrol or metabolites prior to treatment with 10 µM topotecan. The control group was pretreated with vehicle prior to topotecan. *P<0.05 compared with control group. Data represent the mean percentage of cells ± standard error of the mean from 4 separate experiments. PARP, poly ADP-ribose polymerase; R, resveratrol aglycone; R3G, resveratrol-3-O-glucuronide; R4’G, resveratrol-4’-O-glucuronide; R3S, resveratrol-3-O-sulfate.

Figure 5. Co-treatment of Jurkat cells with camptothecin and resveratrol aglycone, but not its metabolites, decreases the percentage of cells with DNA strand breaks, as determined by the terminal deoxynucleotidyl transferase dUTP nick end-labeling assay. (A) Percentage of cells with DNA strand breaks following treatment with 10 µM resveratrol or its metabolites for 4 h without drugs. The control group was treated with 0.1% dimethyl sulfoxide (vehicle). (B) Percentage of cells with DNA strand breaks following treatment with 10 µM resveratrol or its metabolites, plus 5 µM camptothecin. The control group was treated with vehicle and camptothecin. (C) Percentage of cells with DNA strand breaks following treatment with 10 µM resveratrol or its metabolites, plus 10 µM topotecan. The control group was treated with vehicle and topotecan. *P<0.05 compared with control group. Data represent the mean of the percentage of cells ± standard error of the mean from 5 separate experiments. R, resveratrol aglycone; R3G, resveratrol-3-O-glucuronide; R4’G, resveratrol-4’-O-glucuronide; R3S, resveratrol-3-O-sulfate.
resveratrol 4′-O-D-glucuronide (Fig. 5C; P<0.05). Co-treatment with resveratrol 3-O-D-glucuronide or resveratrol-3-O-sulfate did not alter DNA damage induced by camptothecin or topotecan, and no differences in MFI were observed for any of the treatment groups (data not shown).

Discussion

The topoisomerase I inhibitors camptothecin and topotecan were utilized in the present study as a means to induce DNA damage and test the ability of glucuronidated and sulfated metabolites of resveratrol to prevent DNA strand breaks. The pretreatment of Jurkat T cells with resveratrol-3-O-glucuronide and resveratrol-4′-O-glucuronide significantly decreased the mean fluorescence signal from the FITC-UTP used for end-labeling DNA strand breaks induced by camptothecin, suggesting that these resveratrol metabolites were able to reduce the number of strand breaks per cell caused by this drug. No differences in camptothecin- or topotecan-induced DNA damage, as measured by H2AX phosphorylation, were observed between the control and any of the pretreatment groups, with the exception of resveratrol aglycone. DNA strand breaks induce a rapid response by the DNA repair/apoptotic machinery. H2AX is phosphorylated following single-strand DNA breaks and replication stress (by ATR), double-strand DNA breaks (by ATM), and fragmentation of DNA during apoptosis (by DNA-PK) (19,20). It has been reported that the region of phosphorylated H2AX extends up to 1 megabase on either side of a double-strand DNA break in mammalian cells; this post-translational modification is the first step for the recruitment of DNA repair complexes to the damaged sites (19-22). The labeling of DNA ends by the TUNEL assay is a direct method of determining DNA strand breakage and is a more sensitive method, compared with phosphorylated H2AX, for determining the extent of DNA damage on a per-cell basis.

Camptothecin is a lipophilic compound and topotecan is its water-soluble analogue. It has been reported that lipophilic camptothecin has a greater topoisomerase inhibitory effect and cytotoxicity than its water-soluble counterparts (23,24). However, the two compounds demonstrated a similar ability to induce the phosphorylation of H2AX and DNA strand breaks in the present study. Therefore, it remains unclear why pretreatment with the glucuronide metabolites of resveratrol produced a reduction in DNA strand breaks (as determined by the TUNEL assay) following camptothecin, but not topotecan, treatment.

Resveratrol aglycone has been reported to have anticancer and chemosensitizing activities against cancer cells; mechanisms of action for this have previously been proposed (1,25-28). Anticancer mechanisms of action for resveratrol, which have been described predominantly from in vitro studies, include the inhibition of transcription factors, kinases and other molecules involved in cell proliferation and survival. In animal models, resveratrol has demonstrated efficacy against breast, esophageal, lung and colon cancer, and was reported to decrease the extent of metastasis of melanoma and lung or colon carcinoma (29-37). However, resveratrol aglycone has been reported to protect certain types of cells from DNA damage. For example, a previous study demonstrated that resveratrol could inhibit DNA damage in the kidneys of rats treated with the carcinogen KBrO₃ (38). In addition, resveratrol attenuated DNA damage induced by H₂O₂ in glioma cells and peripheral blood lymphocytes, protected DNA from damage by chromium, and reduced the number of DNA adducts induced by the carcinogen dibenzo[a,l]pyrene (39-42). In the present study, the pretreatment of Jurkat cells with 10 µM resveratrol aglycone consistently increased the level of DNA damage induced by camptothecin and topotecan and was used as a positive control in the DNA damage and apoptosis assays. However, a decrease in DNA strand breaks in cells co-treated for 4 h with resveratrol and camptothecin was observed, as in cells co-treated with resveratrol 4′-O-D-glucuronide and topotecan. As has been observed for a number of chemotherapeutic drugs, camptothecins induce oxidative stress in various tissues (43-45); the short co-treatment period with resveratrol in the present study may have provided protective effects against drug-induced oxidative stress due to resveratrol's antioxidant activities (1). Furthermore, camptothecin and resveratrol are lipophilic agents, which may have increased direct interactions between the molecules during co-treatment.

There is limited data available concerning the activity of resveratrol metabolites. Resveratrol-3-O-glucuronide and resveratrol-4′O-glucuronide are two major metabolic products of resveratrol subsequent to ingestion, and the concentrations used in the present study were intended to be physiologically relevant based on a previous study (2). It will be important to understand the potential of these metabolites to protect cells from DNA damage induced by chemotherapeutic drugs. We hypothesize that the effects of these metabolites may be particularly important for the protection of normal cells during chemotherapy. Future research on the protective activities of the metabolic products of resveratrol are necessary to elucidate their interaction with DNA-damaging drugs used in the treatment of cancer. Of note, camptothecins are used in the combinatorial treatment of colorectal cancers (46-48). The increased DNA damage and apoptosis of cells treated with camptothecin and topotecan following pretreatment with resveratrol aglycone in the present study suggests that dietary resveratrol may be a useful addition for treating gastrointestinal cancers that have direct contact with unmetabolized resveratrol.

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References


