DOI:10.1021/jf100702x

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

trans-Resveratrol Reduces Precancerous Colonic Lesions in Dimethylhydrazine-Treated Rats

Irene Alfaras, M. Emília Juan,* and Joana M. Planas

Departament de Fisiologia (Farmàcia) and Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), Universitat de Barcelona, Spain

trans-Resveratrol, a natural occurring polyphenol, has been described as an antiproliferative and proapoptotic agent in vitro. Here, we studied the effect of *trans*-resveratrol administered orally at a dose of 60 mg/kg for 49 days on early preneoplastic markers induced by the intraperitoneal injection of 1,2-dimethylhydrazine (20 mg/kg). We measured *trans*-resveratrol and its derivates by liquid−liquid extraction followed by high-performance liquid chromatography diode array detection analysis in colon contents. Dihydroresveratrol was the most abundant compound in the colon, followed by *trans*-resveratrol glucuronide and small amounts of *trans*-resveratrol and its sulfate. The administration of *trans*-resveratrol decreased aberrant crypt foci by 52%, and mucin depleted foci by 45% in colon. In conclusion, the correlation between the reduction of precancerous colonic lesions and the availability of *trans*-resveratrol in the colon provides a new insight into the therapeutical potential of this polyphenol and its metabolites.

KEYWORDS: Aberrant crypt foci; colon cancer; dihydroresveratrol; mucin-depleted foci; *trans*-resveratrol

INTRODUCTION

trans-Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is synthesized by several plants in response to stress, injury, UV radiation, and fungal infection (*1*). This phytochemical, normally found in dietary products, has been described as a nutraceutic compound with beneficial effects in cancer prevention and treatment. In eliciting these actions, *trans*-resveratrol triggers a variety of cellular and molecular effectors that inhibit the growth of tumor cell lines derived from various human cancers (*2*). Animal and human studies have indicated that oral *trans*-resveratrol has low bioavailability, which may prevent the compound from reaching the target site at therapeutic concentrations in vivo (*1*). This limitation has been attributed to incomplete intestinal absorption (*3*), extensive intestinal metabolism (*4*), and the activity of ABC transporters (*5*). *trans*-Resveratrol enters the enterocyte by passive diffusion. It is metabolized, and its conjugates are secreted back to the intestinal lumen by the members of the ABC family, the Multidrug Resistance Protein 2 (MRP2) and Breast Cancer Resistance Protein (BCRP) (*5*). All of these processes increase the amount of *trans*-resveratrol metabolites reaching the large intestine. They thus favor its potential chemopreventive activity in colon cancer.

The antitumoral activity of *trans*-resveratrol in colon cancer has been studied in vitro (*2*). This phytochemical exerts a large number of effects that interferes with signaling pathways that control cell proliferation. *trans*-Resveratrol inhibits proliferation in colon cell cultures (*6*), which has been attributed to the induction of cell cycle arrest through the inhibition of CDK7 kinase activity (*7*) and to the increasing expression of cyclin A (*8*). In HT-29 cells, the apoptotic effect of *trans*-resveratrol is mediated partly by the intrinsic pathway, through the production of superoxide anions in mitochondria prior to the initiation of the caspase pathway (*6*, *9*). In addition, *trans*-resveratrol also triggers cell death through lysosomes and demonstrates a hierarchy of the proteolytic pathways involved in its cytotoxic mechanism in which lysosomal cathepsin D acts upstream of caspase activation (*10*). Moreover, this polyphenol has been reported to promote apoptosis through the endosplasmic reticulum (*11*) and the induction of CHOP/GADD153 gene expression, which has been acknowledged as a proapoptotic gene (*12*). In contrast, the effects of *trans*-resveratrol on colon cancer in vivo have received little attention (*13*, *14*). Although these studies provide evidence of the activity of *trans*-resveratrol in vivo, they were conducted considering tumors as an end point, without taking into account the early stages of carcinogenesis. Here, we attempt to evaluate the preventive activity of *trans*-resveratrol on the development of markers of colon carcinogenesis. Consequently, rats were given 60 mg/kg of *trans*-resveratrol orally for 49 days. The colon carcinogen 1,2-dimethylhydrazine (DMH) was injected intraperitoneally at days 7, 14, and 21. The chemopreventive activity of *trans*-resveratrol was evaluated by assessing the formation of aberrant crypt foci (ACF) and mucin-depleted foci (MDF) as preneoplastic markers. Moreover, the presence of *trans*-resveratrol and its metabolites in colon was measured. To this end, we developed a method consisting of liquid−liquid extraction followed by high-performance liquid chromatography diode array detection (HPLC-DAD) analysis. The correlation between the chemopreventive activity and the availability of *trans*-resveratrol in the colon provides new insight into the therapeutic potential of this polyphenol and its metabolites.

*To whom correspondence should be addressed. Tel: +34934024505. Fax: +34934035901. E-mail: mejuan@ub.edu.

 Published on Web 06/03/2010

MATERIALS AND METHODS

Chemicals and Reagents. *trans*-Resveratrol was purchased from Second Pharma Co., Ltd. (Shangyu, People's Republic of China), and dihydroresveratrol was from Biopharmalab S.L. (Alicante, Spain). Dose preparation, administration to rats, and sample treatment were performed in dim light to avoid photochemical isomerization of *trans*-resveratrol to the *cis* form. Acetonitrile and methanol were purchased from J. T. Baker (Deventer, Netherlands), and acetic acid was from Scharlau Chemie S.A. (Barcelona, Spain). All of these solvents were HPLC grade. Hydroxypropyl-β-cyclodextrin, β-glucuronidase type L-II (from *Patella vulgata*), and sulfatase type H-1 (from *Helix pomatia*) were from Sigma-Aldrich (St. Louis, MO). Water used in all experiments was passed through a Milli-Q water purification system (18 mΩ) (Millipore, Milan, Italy).

Rats and Diets. Male adult Sprague−Dawley rats (7 weeks old) were housed in cages (n = 3/cage) under controlled conditions of a 12 h light: dark cycle, at a temperature of 22 ± 3 °C and a relative humidity of 40−70%. Water and a standard solid diet (2014 Teklad Global 14%, Harlan, Spain) were consumed ad libitum. No traces of *trans*-resveratrol were detected in the commercial diet or in the plasma of control rats, analyzed following Juan et al. (*15*). Handling and killing of rats were in full accordance with the European Community guidelines for the care and management of laboratory rats. The studies were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona (ref 2269/01). All rat manipulations were carried out in the morning to minimize the effects of circadian rhythm.

Experimental Design. After 1 week of acclimatization, rats were stratified by body weight and assigned to 1 of 3 experimental groups (n = 6 rats/group) such that there were no differences in mean initial weight. The experimental groups were as follows: control group (no test agent and no carcinogen), the DMH group (no test agent and DMH), and the resveratrol group (*trans*-resveratrol and DMH). Rats were orally administered by gavage at a constant volume of 10 mL/kg every day for 49 days. *trans*-Resveratrol was administered at a dose of 60 mg/kg considering this compound as a potential nutraceutical. Because of its low solubility in water, *trans*-resveratrol was dissolved in 20% hydroxypropyl-β-cyclodextrin (v/v). Rats in the control and DMH groups were given only the solvent during the same period. At days 7, 14, and 21, rats from DMH and resveratrol groups received an intraperitoneal injection of carcinogen (20 mg DMH/kg dissolved in EDTA 1 mmol/L, pH 6.5), and control animals received only the solvent at a constant volume of 1 mL/kg. Oral and intraperitoneal doses were adjusted according to rat weight to ensure a constant dose, and they were freshly prepared immediately before each administration.

Body weight and food and water consumption were monitored daily. The feed conversion efficiency (FCE) was calculated as the weekly body weight gain divided by the food consumption.

Sample Collection. At the end of the study, rats were deprived of food overnight and anesthetized with ketamine (90 mg/kg) and xylacine (10 mg/kg). Blood samples were collected by cardiac puncture and transferred as follows: 1 mL into EDTA-K_3 for hematology, 2 mL into a tube without anticoagulant for clinical chemistry, and 1 mL into EDTA-K_3 for *trans*-resveratrol determination. Serum for clinical chemistry and plasma for *trans*-resveratrol analysis were obtained after centrifugation of blood samples at 1500*g* (MEGAFUGE 1.0R, Heraeus, Boadilla, Spain) for 15 min at 4 °C.

A gross necropsy was performed. Subsequently, the brain, lungs, spleen, heart, liver, kidney, and testicle were excised and trimmed of any adherent tissue, and their wet weights were immediately recorded to avoid drying. Results are expressed as organ weight relative to 100 g of body weight (%).

The colon was removed, and the contents were collected and stored at −20 °C for extraction and subsequent analysis of *trans*-resveratrol. The colon was then washed in phosphate-buffered solution (PBS) (pH 7.4) and trimmed of adherent mesenteric tissue, and its wet weight was recorded. The colon was divided into three equal segments: proximal (close to the cecum), medial, and distal (close to the rectum). Each segment was opened along the longitudinal median and pinned flat onto a polystyrene board for the ACF assay.

Hematology and Clinical Chemistry. Complete and differential cell counts were performed using a Cell-Dyn blood analyzer (Abbott Diagnostics Division, Santa Clara, CA). Biochemical analyses of serum were performed with a Roche/Hitachi 747 clinical analyzer from Roche Diagnostics GmbH (Mannheim, Germany).

Determination of *trans*-Resveratrol and Its Metabolites in Plasma. *trans*-Resveratrol and glucuronide and sulfate conjugates concentrations were determined using the method of Juan et al. (*15*) in plasma samples 24 h after the last oral administration.

Quantification of *trans*-Resveratrol and Its Metabolites in Colon Content. Colon content samples were defrosted at room temperature and weighed. To extract *trans*-resveratrol, 10 mL of 80% methanol with 2.5% acetic acid and 10 μL of 15% ascorbic acid were added to 1 g of colon sample. The mixture was agitated with constant stirring for 30 min at 60 °C. Then, samples were transferred to a centrifuge tube, and the content remaining in the beaker was collected with an additional 2 mL of acidified methanol. The homogenates were centrifuged at 33000*g* (Centrikon H-401, Kontron Hermle Instruments, Italy) for 30 min at 4 °C. The supernatant was transferred to a clean tube, and the residue was extracted once again, by the same procedure. The organic solvent of the supernatant was evaporated with a Concentrator 5301 (Eppendorf Iberica, S.L., San Sebastian de los Reyes, Spain) at 45 °C to a final volume of 500 μL.

The determination of *trans*-resveratrol and its metabolites by HPLC analysis was carried out following Juan et al. (*15*) but with a different gradient elution program. The execution was performed as follows: 0−5 min, 15% B; 7 min, 20% B; 10 min, 21% B; 20 min, 22% B; 30 min, 30% B; 35 min, 35% B; 40 min, 40% B; 45 min, 50% B; 50 min, 70% B; 55−60 min, 100% B; and 62 min, 15% B. There was a 5 min delay prior to the injection of the next sample to ensure re-equilibration of the column.

The chromatograms were obtained at 306 and 276 nm, which correspond to the maximum absorbance of *trans*-resveratrol and dihydroresveratrol, respectively. Authentic standards of *trans*-resveratrol and dihydroresveratrol were used for the construction of calibration curves. The chromatographic peaks were further identified by spectroscopic analysis with diode array-UV detection from 220 to 400 nm. The results of the analyses are expressed as nmol/L of plasma and nmol/g of colonic content.

The identity of the peaks detected in colon content was confirmed by mass spectrometry. An Agilent series 1100 HPLC instrument with an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, PE Sciex, Concord, Ontario, Canada) equipped with a Turbo IonSpray source in negative-ion mode was used for HPLC-MS analysis. The ion spray voltage was 3500 V, using nitrogen as the nebulizer gas (10 arbitrary units) and curtain gas (15 arbitrary units). The detection conditions were optimized with a standard solution of *trans*-resveratrol and dihydroresveratrol in the presence of LC mobile phase, as follows: declustering potential, −70 V; focusing potential, −200 V; drying gas (N_2) heated to 400 °C and introduced at a flow rate of 5000 cm^3/min. Mass spectra were acquired in the 100−500 m/z range.

Validation of the Method To Quantify *trans*-Resveratrol in Colon Content. The method was validated according to The United States Pharmacopoeia (*16*). Approximately 1 g of blank colon content was spiked with 5 nmol of *trans*-resveratrol and agitated in the vortex for 2 min before being processed as indicated above. Precision was determined by assaying six samples at 5 nmol/g and was expressed as the experimental coefficient of variation (CV). Recovery was measured by spiking blank samples with a final concentration of 5 nmol/g (n = 6). Finally, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated by measuring the analytical background response of six blanks of colon content. Signal-to-noise ratios of 3:1 and 10:1 were used for the LOD and LOQ, respectively.

ACF. Aberrant crypts (AC) and ACF were evaluated using a modification of a method previously described (*17*). Briefly, the intestine was fixed in 10% buffered formalin, pH 7.4 (Sigma-Aldrich), for a minimum of 24 h. The fixed segments were washed in PBS. Colon segments were stained in a 0.2% methylene blue solution for 8−10 min, and the excess of dye was rinsed off with PBS. Each segment was placed mucosal side up on a microscopic slide and examined by light microscope at 20× magnification (model CHS; Olympus Optical Co., Ltd., Hamburg, Germany). ACF were identified as described elsewhere (*17*). The number of ACF and AC in each focus was counted. The scores were checked by an observer who was blinded to treatment groups. All of the images of the mucosal surface were captured with KAPPA Image Base Control 2.6 (KAPPA opto-electronics GmbH, Gleichen, Germany).

Mucin-Depleted Foci. After ACF determination, colons were kept in 10% buffered formalin, pH 7.4, at −4 °C and later processed with the high-iron diamine Alcian blue staining (HID-AB) at pH 2.5 for the visualization of mucin production. Segments were washed in PBS and then dyed in a high-iron diamine solution for 18−24 h protected from the light. Then, segments were washed and stained for 5 min with 1% alcian blue in 3% acetic acid, washed again, and finally stained for 2 min in neutral red (1 g of neutral red dissolved in 1 L of mQ water with 2 mL of 1% acetic acid). The HID-AB-stained colons were placed mucosal side up in slides and observed under a light microscope at 20× magnification. The total number of MDF and number of mucin-depleted aberrant crypts (MDAC) per focus in colon were determined following Caderni et al. (*18*): absence or little production of mucins, distortion of the opening of the lumen as compared with normal surrounding crypts, and elevation of the lesion above the surface of the colon. The scores were determined by an observer blinded to the experiment. Images of the mucosal surface were captured with KAPPA Image Base Control 2.6.

Statistical Analysis. Results are presented as the means ± standard errors of the mean (SEMs). All data evaluation and analyses were done by GraphPad Prism 4 (GraphPad Software, Inc., La Joya, CA). Organs, colon weight, and hematological and clinical biochemistry were compared using one-way analysis of variance (ANOVA), followed by Bonferroni's posthoc test. When the normality test was significant, as evaluated by the Kolmogorov−Smirnov test, differences between means were assessed instead by the nonparametric Kruskal−Wallis test. Significant differences of body weight, FCE, the number ACF per segment, and the number of AC per ACF were analyzed by two-way ANOVA followed by Bonferroni's posthoc test. The total number of AC, ACF, MDF, and MDAC was compared using Student's unpaired t test. The F test was performed to check that samples had equal variances. For all tests, $P < 0.05$ was considered significant.

RESULTS

Body Weight, Food, and Water Consumption. No mortality or adverse effects occurred during the experiment. Stool consistency was firm (pelleted) throughout the study, with no visible differences between groups. Body weight was not affected by the administration of DMH or *trans*-resveratrol with respect to the control group. Thus, the body weight of the control animals increased from 235 ± 16 g on day 1 to 376 ± 39 g on day 49. The DMH group increased from 232 ± 13 g on day 1 to 353 ± 29 g on day 49, and the resveratrol group increased from 235 ± 20 g on day 1 to 354 ± 34 g on day 49. There were no significant differences in food or water consumption between the three groups. Feed conversion efficiency (**Figure 1**) was highest during the first week, decreased during the second and third week, and remained constant thereafter, with no significant differences between groups.

Gross Necropsy. At the end of the study, a postmortem examination showed no evidence of gross abnormality or toxicity in any group. The examination of the vital organs carried out during the autopsy did not show macroscopic differences in size, color, or texture in any of the groups studied. The final relative weights of liver, kidney, heart, brain, lungs, testicles, spleen, and colon were not different among groups.

Hematology and Clinical Chemistry. The results of the hematologic tests carried out at the end of the study did not show any differences among control, DMH, or resveratrol groups. The erythrocyte, leukocyte, and platelets results were comparable to those obtained previously (*19*). The clinical chemistry variables were also evaluated. No differences between groups were observed in glucose or protein concentrations. Serum cholesterol, triglycerides, and high-density lipoproteins were not affected by the oral administration of 60 mg/kg of *trans*-resveratrol or DMH treatment. The hepatic integrity was maintained throughout the experiment as indicated with ALT and AST, which did not differ between groups. Renal function and the plasma levels of electrolytes were within reference values.

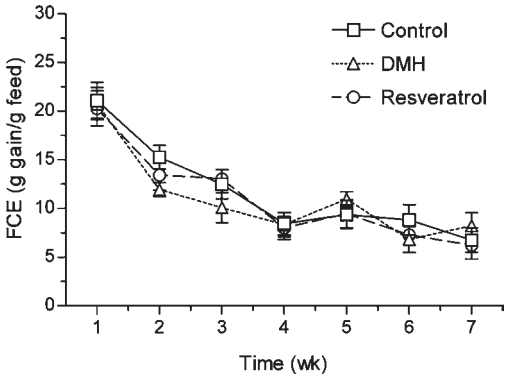

Figure 1. FCE of male Sprague−Dawley rats of control (no test agent and no carcinogen), DMH (no test agent and 20 mg/kg DMH once a week for 3 weeks), and resveratrol groups (60 mg/kg *trans*-resveratrol and 20 mg/kg DMH once a week for 3 weeks). Results are expressed as means ± SEMs, $n = 6$. No differences between groups are found. Differences over time: control group, 1 week = 2 weeks > 3 weeks = 4 weeks = 5 weeks = 6 weeks = 7 weeks; 2 weeks = 3 weeks = 5 weeks; DMH group, 1 week > 2 weeks = 3 weeks = 4 weeks = 5 weeks = 6 weeks = 7 weeks; and *trans*-resveratrol group, 1 week > 2 weeks = 3 weeks = 5 weeks > 4 weeks = 6 weeks = 7 weeks; 3 weeks = 6 weeks; 4 weeks = 5 weeks = 6 weeks = 7 weeks, $p < 0.05$.

Determination of *trans*-Resveratrol and Its Metabolites in Plasma. Free *trans*-resveratrol was the main compound present in plasma 24 h after the last administration, with concentrations of 43.2 ± 10.8 nmol/L. *trans*-Resveratrol glucuronide was identified in plasma of only two of the six *trans*-resveratrol-treated rats, at a concentration of 37.8 ± 13.8 nmol/L. No traces of sulfate conjugate were found.

Validation of the Method To Quantify *trans*-Resveratrol in Colon Content. The extraction of *trans*-resveratrol and its metabolites in colon content was attempted using different solvents. When ethanol, ethyl acetate, and methanol (80%, v/v) without acidification were evaluated, the recovery was lower than that obtained when methanol was mixed with acetic acid (2.5%). The mean total recovery was 98.3 ± 11.3%, which indicates that *trans*-resveratrol was quantitatively extracted by this method. The precision expressed as CV was 13.5%, which is less than the recommended value of 20% (*20*). The sensitivity was adequate to measure *trans*-resveratrol in colonic content samples with an LOD of 16.5 pmol/g and LOQ of 20.6 pmol/g. The chromatographic gradient elution, which was modified from a previous method (*15*), allowed a good separation of *trans*-resveratrol and its glucuronide and sulfate conjugates from interference peaks with detection at 306 nm. Moreover, the method allowed the identification and quantification of dihydroresveratrol at 276 nm.

Quantification of *trans*-Resveratrol and Its Metabolites in Colon Content. The method developed allowed the separation and identification of *trans*-resveratrol (peak 1) and its glucuronide and sulfate conjugates (peaks 2 and 3, respectively) (**Figure 2**). The identity of the peaks was confirmed by MS. *trans*-Resveratrol glucuronide was characterized by the deprotonated molecular ion $(M - H)^-$ at m/z 403 (**Figure 2C**), whereas the resveratrol fragment was observed at m/z 227. The sulfate conjugate showed a deprotonated molecular ion $(M - H)^-$ at m/z 307 (**Figure 2C**) and also the resveratrol fragment at m/z 227. The method also allowed identification of another metabolite, dihydroresveratrol, resulting from the reduction of the double bond (**Figure 3**). This compound eluted at 28 min, which was barely 1 min after *trans*-resveratrol, but it had a different spectrum and maximum of absorbance (λ_{max} 276 nm) (**Figure 3A**). Dihydroresveratrol was further identified

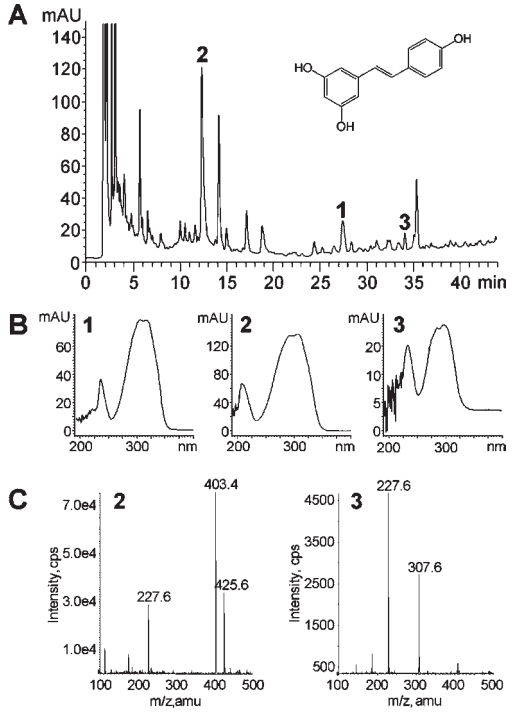

Figure 2. HPLC chromatogram and mass spectra of *trans*-resveratrol obtained in colonic content from rats of the resveratrol group, 24 h after the last oral administration of 60 mg/kg. (**A**) HPLC chromatogram at 306 nm. Peaks of (1) *trans*-resveratrol and its conjugates, (2) glucuronide, and (3) sulfate are indicated. (**B**) UV spectrum obtained by diode array detection of (1) *trans*-resveratrol, (2) glucuronide, and (3) sulfate. (**C**) Full-scan product ion mass spectra of (2) glucuronide and (3) sulfate.

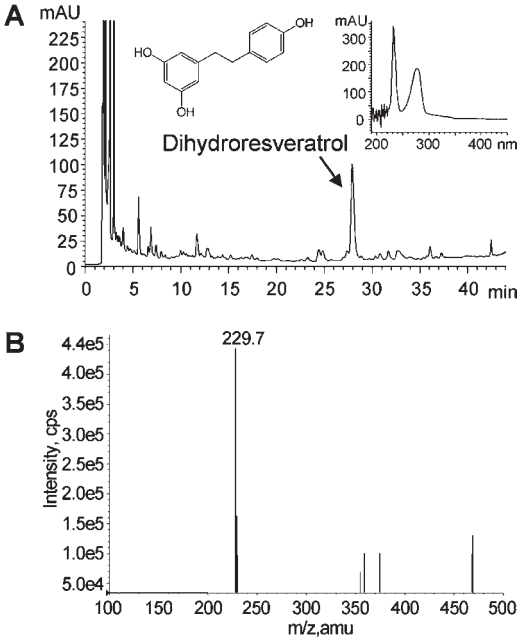

Figure 3. HPLC chromatogram and mass spectra of dihydroresveratrol obtained in colonic content from rats of the resveratrol group, 24 h after the last oral administration of 60 mg/kg. (**A**) HPLC chromatogram at 276 nm. Inserts depict the chemical structure of dihydroresveratol and its UV spectrum obtained by diode array detection. (**B**) Full-scan product ion mass spectra of dihydroresveratrol.

by MS, which gave a deprotonated molecular ion $(M - H)^-$ at m/z 229 (**Figure 3B**).

trans-Resveratrol was detected in the colon content of rats treated with 60 mg/kg, 24 h after the last administration, at a concentration of 0.68 ± 0.24 nmol/g. The most abundant metabolite in colon was dihydroresveratrol, with a concentration of 303.0 ± 34.7 nmol/g, 446-fold that of the parent compound. The glucuronide and sulfate conjugates of *trans*-resveratrol were also detected, at concentrations of 3.40 ± 1.29 and 0.44 ± 0.23 nmol/g, respectively.

ACF. Control rats showed no microscopically observable changes in colon morphology. DMH-injected rats developed ACF (**Figure 4**) with the absence of lesions in the proximal colon followed by a few ACF in the medial segment and more in the distal colon, in all groups (**Table 1**). *trans*-Resveratrol treatment inhibited the number of ACF by 58 ($p < 0.01$) and 48% ($p < 0.001$) in the medial and distal segments, respectively. Crypt multiplicity (number of AC per ACF) was also counted (**Figure 4I**) and showed the same pattern, more singlets than 2, 3, or ≥4 AC in both groups. However, rats treated with *trans*-resveratrol had fewer foci with 1 ($p < 0.001$), 2 ($p < 0.01$), and 3 crypts being inhibited by 53, 49, and 64%, respectively. *trans*-Resveratrol treatment also decreased the number of total AC (**Table 1**) present in colon by 50% ($p < 0.01$).

Mucin-Depleted Foci. MDF were observed in the DMH-treated groups (**Figure 4**). The daily oral administration of 60 mg/kg of *trans*-resveratrol reduced the number of MDF by 45% ($p < 0.05$) (**Figure 4H**), with 5.00 ± 0.01 MDF in the DMH group and 2.75 ± 0.75 MDF in the resveratrol group. *trans*-Resveratrol treatment reduced the number of MDF lesions by 36 and 53% in the medial and distal segment, respectively (**Table 1**). The number of foci with 1, 2, 3, and ≥4 AC did not differ between groups, but there was a reduction of 48% in the total number of MDAC ($p < 0.05$) in the resveratrol group with respect to the DMH group (**Table 1**).

DISCUSSION

Colorectal cancer is one of the leading causes of death in both men and women in Western countries, and it is usually lethal when diagnosed at later stages of progression (*21*). Environmental aspects are believed to be involved in colon carcinogenesis, including diet. Up to 80% of sporadic colorectal cancers are therefore potentially preventable (*22*). Diet and lifestyle are most likely related to colon cancer etiology through the overconsumption of energy, coupled with inadequate intake of protective substances, including micronutrients, dietary fiber, and a variety of phytochemicals (*23*). For this reason, the present study aims to evaluate the effect of *trans*-resveratrol on the development of early markers of colon carcinogenesis in DMH-treated rats and measure the concentrations of this polyphenol and its metabolites in colon content.

Our results show that the method presented is suitable for the extraction, identification, and quantification of the target substance and its metabolites, given that we obtained a recovery of 98%, good precision, and adequate sensitivity. Once the method was established, it was applied to the samples of colon content obtained 24 h after the last oral administration of 60 mg/kg of *trans*-resveratrol. Our results showed that this compound reached the colon together with its metabolites. The most abundant compound was dihydroresveratrol followed by resveratrol glucuronide and minor amounts of *trans*-resveratrol and its sulfate conjugate. The conjugates come from the metabolism in the enterocyte and subsequent extrusion back to the intestinal lumen (*4*, *5*). Moreover, enterohepatic circulation may affect the concentration of these compounds in the colon (*24*). Although these conjugates are believed to be pharmacologically inactive and excreted in urine and bile, deconjugation has been reported to

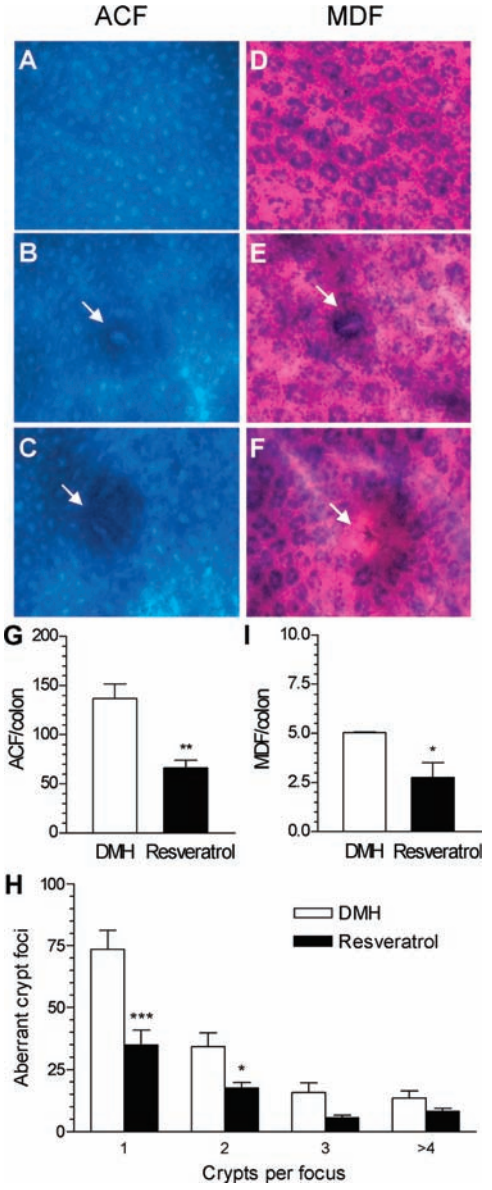

Figure 4. Preneoplastic lesions ACF (**A**−**C**) and MDF (**D**−**F**) observed under a light microscope after staining of the colon with methylene blue or HID-AB, respectively. Images show the whole mount colon of control rats (**A** and **D**) and DMH-treated animals depicting a topographic view of ACF with 1 crypt (**B**) and more than 3 crypts (**C**), a mucinous ACF (**E**), and a mucin-depleted foci (**F**). Effects of *trans*-resveratrol on the total number of ACF (**G**), crypt multiplicity of ACF (**H**), and total number of mucin-depleted foci (**I**). Rats were orally administered with 60 mg/kg *trans*-resveratrol for 49 days. DMH (20 mg/kg) was injected intraperitoneally at days 7, 14, and 21. Results are expressed as means + SEMs ($n = 6$); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs DMH group. Differences in the number of ACs per focus (**H**): DMH group, 1 AC/ACF > 2 AC/ACF > 3 AC/ACF = >4 AC/ACF; resveratrol group, 1 AC/ACF > 2 AC/ACF = 3 AC/ACF = >4 AC/ACF, $p < 0.05$.

occur in the gut, releasing the parent compound (*25*, *26*). Consequently, the glucuronide and sulfate conjugates of *trans*-resveratrol could act as colon-specific prodrugs, as shown for sulfate-conjugated methylprednisolone and prednisolone. The latter had been developed to release the corresponding glucocorticoid in the cecum for the treatment of inflammatory bowel disease (*26*).

The method also permitted the detection of dihydroresveratrol, a metabolite of *trans*-resveratrol, which is formed by

Table 1. Effect of *trans*-Resveratrol in the Occurrence of Preneoplastic Lesions in Colon Induced by DMH[a]

	DMH group	resveratrol group
ACF/proximal	0	0
ACF/medial	52.8 ± 13.3	22.2 ± 3.94**
ACF/distal	84.2 ± 8.82	44.0 ± 9.48***
AC/colon	218 ± 28.9	110 ± 13.8**
MDF/proximal	0	0
MDF/medial	2.33 ± 0.88	1.50 ± 0.29
MDF/distal	2.67 ± 0.88	1.25 ± 0.75
MDAC/colon	10.7 ± 0.33	5.50 ± 1.67*

[a] Values are means ± SEMs, $n = 6$. * Significantly different from DMH group, $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

the reduction of the stilbenic double bond. This compound is scarcely known, and its presence has been reported only in urine but not in plasma (*27*, *28*). In humans, it was detected as dihydroresveratrol glucuronide and sulfate (*27*) and in rats as dihydroresveratrol and its sulfate conjugate (*28*). These authors attributed the presence of dihydroresveratrol to the activity of the intestinal microflora and posterior absorption. That hypothesis is consistent with our results, since when intestinal perfusions were performed in the absence of bacteria only resveratrol glucuronide and sulfate were found (*5*). A recent study identified dihydroresveratrol as a metabolite produced by *E. lenta* and *B. uniformis*, both of which have been isolated from human feces (*29*). All of these findings support the involvement of the intestinal flora in the synthesis of this metabolite. The biological activity of dihydroresveratrol is hardly known, although it has been reported to possess less antioxidant activity and less ability to inhibit DNA synthesis (*30*). However, it has a slightly stronger effect than *trans*-resveratrol in cell growth inhibition assays (*31*).

In the present study, we evaluate the potential chemopreventive activity of *trans*-resveratrol on colon cancer in the DMH rat model, where carcinogenesis develops through a multistep process as it does in humans. ACF have been identified in humans at high risk and are widely used as a surrogate marker of colon cancer (*32*). Prior to the performance of our study, we optimized the experimental conditions (data not shown). Therefore, we assayed two and three subcutaneous injections of DMH (20 mg/kg, 1 week apart) and different observation periods to validate the appearance of ACF. Our results showed that three subcutaneous injections followed by an observation period of 4 weeks proved to be appropriate for the screening of potentially chemopreventive agents. Under our experimental conditions, *trans*-resveratrol and/or its metabolites reduced the number of preneoplastic lesions, since ACF were inhibited by 52% and the total number of AC by 50%. Furthermore, MDF were described in carcinogen-treated rodents (*18*) and in humans at high risk of colon cancer (*32*). MDF are characterized by harboring mutations that show Wnt signaling activation like in colon tumors, suggesting that these lesions are precancerous (*32*). In our experimental condition, the number of MDF was reduced by 50%, thus remarking the protecting activity exerted by *trans*-resveratrol in the colon mucosa. Because *trans*-resveratrol was administered 1 week prior to the first exposure to DMH, the present results demonstrated that *trans*-resveratrol acts as an efficient agent inhibiting cancer initiation.

The potential cancer chemopreventive activity of *trans*-resveratrol in vivo has been examined only in long-term studies (*13*, *14*, *33*−*35*). The effect of *trans*-resveratrol on azoxymethane-induced colon carcinogenesis was assessed in F344 rats. This phytochemical was administered in drinking water at a dose of 200 μg/kg for 100 days, beginning 10 days before the administration of the carcinogen (*33*).

trans-Resveratrol reduced the growth of colorectal ACF modulating the expression of bax and p21, both involved in the regulation of cell proliferation and apoptosis (*33*). Sengottuvelan et al. also assessed the anticarcinogenic activity of 8 mg/kg of *trans*-resveratrol in a model of colon carcinogenesis induced by DMH, but these were long-term experiments that lasted 30 weeks (*14*, *34*, *35*). Their results showed that in rats, *trans*-resveratrol markedly reduced the number of DMH-induced ACF and incidence and size of tumors, possibly through the modulation of antioxidant defense status and activities of carcinogen-detoxifying enzymes (*14*, *34*, *35*). In Apc^{Min} mice, which are used as a model of human familial adenomatous polyposis, *trans*-resveratrol administered in drinking water at a dose of 15 mg/kg for 7 weeks prevented the formation of colon tumors and reduced the formation of small intestinal tumor by 70% by down-regulating genes that are directly involved in cell cycle progression such as cyclin D1, D2, and DP-1 and in the inhibition of the carcinogenic process and tumor expansion (*13*). It is noteworthy that using the same animal model where *trans*-resveratrol was administered in the diet at 4, 20, or 90 mg/kg for 7 weeks in Apc^{Min}, there were no effects in colon tumorigenesis (*36*). Those results are in accordance with the ones obtained by Sale et al. (*37*), who only found a decrease on the number of adenomas when doses of 280 mg/kg were used. The effect of the higher dose of *trans*-resveratrol was associated with inhibition of COX enzymes and interference with prostaglandin E_2 generation (*37*).

Our findings showed that *trans*-resveratrol given orally significantly reduced DMH-induced colon preneoplastic lesions without any apparent adverse effects. For chemopreventive purposes, daily, long-term ingestion is necessary, so safety was a prime consideration. Our results indicate that in terms of body growth, *trans*-resveratrol is well-tolerated by animals, as shown previously (*19*). Moreover, the gross necropsy revealed a normal appearance of the vital organs without the presence of pathological lesions. These results were substantiated by the results of the hematological and clinical chemistry studies, which indicated that *trans*-resveratrol is nontoxic (*19*).

In conclusion, these results indicate that following the oral administration of 60 mg/kg of *trans*-resveratrol, some of this polyphenol and its metabolites reached the colon. It inhibited ACF formation and mucin-depleted foci. The present findings support the hypothesis that *trans*-resveratrol contributes to the prevention of colon carcinogenesis and provides an insight into the extensive metabolism that this polyphenol undergoes in the intestine.

ABBREVIATIONS USED

AC, aberrant crypt; ACF, aberrant crypt foci; BCRP, Breast Cancer Resistance Protein; DMH, 1,2-dimethylhydrazine; FCE, food conversion efficiency; MDAC, mucin-depleted aberrant crypt; MDF, mucin-depleted foci; MRP2, Multidrug Resistance Protein 2.

LITERATURE CITED

(1) Baur, J. A.; Sinclair, D. A. Therapeutic potential of resveratrol: the in vivo evidence. *Nat. Rev. Drug Discovery* **2006**, *5*, 493–506.

(2) Bishayee, A. Cancer prevention and treatment with resveratrol: From rodent studies to clinical trials. *Cancer Prev. Res.* (*Philadelphia, PA*) **2009**, *2*, 409–418.

(3) Kuhnle, G.; Spencer, J. P.; Chowrimootoo, G.; Schroeter, H.; et al. Resveratrol is absorbed in the small intestine as resveratrol glucuronide. *Biochem. Biophys. Res. Commun.* **2000**, *272*, 212–217.

(4) Sabolovic, N.; Humbert, A. C.; Radominska-Pandya, A.; Magdalou, J. Resveratrol is efficiently glucuronidated by UDP-glucuronosyltransferases in the human gastrointestinal tract and in Caco-2 cells. *Biopharm. Drug Dispos.* **2006**, *27*, 181–189.

(5) Juan, M. E.; González-Pons, E.; Planas, J. M. Multidrug resistance proteins restrain the intestinal absorption of *trans*-resveratrol. *J. Nutr.* **2010**, *140*, 489–495.

(6) Juan, M. E.; Wenzel, U.; Daniel, H.; Planas, J. M. Resveratrol induces apoptosis through ROS-dependent mitochondria pathway in HT-29 human colorectal carcinoma cells. *J. Agric. Food Chem.* **2008**, *56*, 4813–4818.

(7) Liang, Y. C.; Tsai, S. H.; Chen, L.; Lin-Shiau, S. Y.; Lin, J. K. Resveratrol-induced G2 arrest through the inhibition of CDK7 and p34CDC2 kinases in colon carcinoma HT29 cells. *Biochem. Pharmacol.* **2003**, *65*, 1053–1060.

(8) Colin, D.; Gimazane, A.; Lizard, G.; Izard, J. C.; et al. Effects of resveratrol analogs on cell cycle progression, cell cycle associated proteins and 5-fluorouracil sensitivity in human derived colon cancer cells. *Int. J. Cancer* **2009**, *124*, 2780–2788.

(9) Hwang, J. T.; Kwak, D. W.; Lin, S. K.; Kim, H. M.; et al. Resveratrol induces apoptosis in chemoresistant cancer cells via modulation of AMPK signaling pathway. *Ann. N.Y. Acad. Sci.* **2007**, *1095*, 441–448.

(10) Trincheri, N. F.; Nicotra, G.; Follo, C.; Castino, R.; Isidoro, C. Resveratrol induces cell death in colorectal cancer cells by a novel pathway involving lysosomal cathepsin D. *Carcinogenesis* **2007**, *28*, 922–931.

(11) Park, J. W.; Woo, K. J.; Lee, J. T.; Lim, J. H.; et al. Resveratrol induces pro-apoptotic endoplasmic reticulum stress in human colon cancer cells. *Oncol. Rep.* **2007**, *18*, 1269–1273.

(12) Woo, K. J.; Lee, T. J.; Lee, S. H.; Lee, J. M.; et al. Elevated gadd153/chop expression during resveratrol-induced apoptosis in human colon cancer cells. *Biochem. Pharmacol.* **2007**, *73*, 68–76.

(13) Schneider, Y.; Duranton, B.; Gossé, F.; Schleiffer, R.; et al. Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis. *Nutr. Cancer* **2001**, *39*, 102–107.

(14) Sengottuvelan, M.; Viswanathan, P.; Nalini, N. Chemopreventive effect of *trans*-resveratrol, a phytoalexin against colonic aberrant crypt foci and cell proliferation in 1,2-dimethylhydrazine induced colon carcinogenesis. *Carcinogenesis* **2006**, *27*, 1038–1046.

(15) Juan, M. E.; Maijó, M.; Planas, J. M. Quantification of *trans*-resveratrol and its metabolites in rat plasma and tissues by HPLC. *J. Pharm. Biomed. Anal.* **2010**, *51*, 391–398.

(16) Validation of compendial methods. In *The United States Pharmacopeia and The National Formulary*, USP32-NF27; The United States Pharmacopeial Convention: Rockville, MD, 2009; pp. 733−736.

(17) Bird, R. P. Observation and quantification of aberrant crypts in the murie colon treated with a colon carcinogen: Preliminary findings. *Cancer Lett.* **1987**, *37*, 147–151.

(18) Caderni, G.; Femia, A. P.; Giannini, A.; Favuzza, A.; et al. Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: Correlation with carcinogenesis. *Cancer Res.* **2003**, *63*, 2388–2392.

(19) Juan, M. E.; Vinardell, M. P.; Planas, J. M. The daily oral administration of high doses of *trans*-resveratrol to rats for 28 days is not harmful. *J. Nutr.* **2002**, *132*, 257–260.

(20) Bansal, S.; DeStefano, A. Key elements of bioanalytical method validation for small molecules. *AAPS J.* **2007**, *9*, E109–E114.

(21) Ferlay, J.; Autier, P.; Boniol, M.; Heanue, M.; et al. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann. Oncol.* **2007**, *18*, 581–592.

(22) Cummings, J. H.; Bingham, S. A. Diet and the prevention of cancer. *BMJ* **1998**, *317*, 1636–1640.

(23) Watson, A. J. An overview of apoptosis and the prevention of colorectal cancer. *Crit. Rev. Oncol. Hematol.* **2006**, *57*, 107–121.

(24) Marier, J. F.; Vachon, P.; Gritsas, A.; Zhang, J.; et al. Metabolism and disposition of resveratrol in rats: Extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. *J. Pharmacol. Exp. Ther.* **2002**, *302*, 369–373.

(25) Sakamoto, H.; Yokota, H.; Kibe, R.; Sayama, Y.; Yuasa, A. Excretion of bisphenol A-glucuronide into the small intestine and deconjugation in the cecum of the rat. *Biochim. Biophys. Acta* **2002**, *1573*, 171–176.

(26) Kong, H.; Kim, Y.; Lee, Y.; Choi, B. Sulfate-conjugated methylprednisolone: Evaluation as a colon-specific methylprednisolone

prodrug and comparison with sulfate-conjugated prednisolone and dexamethasone. *J. Drug Target* **2009**, *17*, 159–167.
(27) Walle, T.; Hsieh, F.; DeLegge, M. H.; Oatis, J. E., Jr.; Walle, U. K. High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos.* **2004**, *32*, 1377–1382.
(28) Wang, D.; Hang, T.; Wu, C.; Liu, W. Identification of the major metabolites of resveratrol in rat urine by HPLC-MS/MS. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2005**, *829*, 97–106.
(29) Jung, C. M.; Heinze, T. M.; Schnackenberg, L. K.; Mullis, L. B.; et al. Interaction of dietary resveratrol with animal-associated bacteria. *FEMS Microbiol. Lett.* **2009**, *297*, 266–273.
(30) Stivala, L. A.; Savio, M.; Carafoli, F.; Perucca, P.; et al. Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. *J. Biol. Chem.* **2001**, *276*, 22586–22594.
(31) Cardile, V.; Lombardo, L.; Spatafora, C.; Tringali, C. Chemoenzymatic synthesis and cell-growth inhibition activity of resveratrol analogues. *Bioorg. Chem.* **2005**, *33*, 22–33.
(32) Femia, A. P.; Caderni, G. Rodent models of colon carcinogenesis for the study of chemopreventive activity of natural products. *Planta Med.* **2008**, *74*, 1602–1607.
(33) Tessitore, L.; Davit, A.; Sarotto, I.; Caderni, G. Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21(CIP) expression. *Carcinogenesis* **2000**, *21*, 1619–1922.
(34) Sengottuvelan, M.; Deeptha, K.; Nalini, N. Influence of dietary resveratrol on early and late molecular markers of 1,2-dimethylhydrazine-induced colon carcinogenesis. *Nutrition* **2009**, *25*, 1169–1176.
(35) Sengottuvelan, M.; Nalini, N. Dietary supplementation of resveratrol suppresses colonic tumour incidence in 1,2-dimethylhydrazine-treated rats by modulating biotransforming enzymes and aberrant crypt foci development. *Br. J. Nutr.* **2006**, *96*, 145–153.
(36) Ziegler, C. C.; Rainwater, L.; Whelan, J.; McEntee, M. F. Dietary resveratrol does not affect intestinal tumorigenesis in Apc(Min/+) mice. *J. Nutr.* **2004**, *134*, 5–10.
(37) Sale, S.; Tunstall, R. G.; Ruparelia, K. C.; Potter, G. A.; et al. Comparison of the effects of the chemopreventive agent resveratrol and its synthetic analog trans 3,4,5,4′-tetramethoxystilbene (DMU-212) on adenoma development in the Apc(Min+) mouse and cyclooxygenase-2 in human-derived colon cancer cells. *Int. J. Cancer* **2005**, *115*, 194–201.

Received for review February 19, 2010. Revised manuscript received May 14, 2010. Accepted May 23, 2010. Supported by the Ministerio de Ciencia y Tecnología Grants AGL2005-05728 and AGL2009-12866 and the Generalitat de Catalunya Grants 2005-SGR-00632 and 2009-SGR-00471. We thank the staff of the Clinical Analysis department of CAP Just Oliveras (Hospitalet de Llobregat, Spain) for excellent technical assistance.