HIGH ABSORPTION BUT VERY LOW BIOAVAILABILITY OF ORAL RESVERATROL IN HUMANS

Thomas Walle, Faye Hsieh, Mark H. DeLegge, John E. Oatis, Jr., and U. Kristina Walle

Department of Cell and Molecular Pharmacology and Experimental Therapeutics (T.W., J.E.O., U.K.W.) and Digestive Disease Center (M.H.D.), Medical University of South Carolina, Charleston, South Carolina; and Amgen, Inc., Thousand Oaks, California (F.H.)

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ABSTRACT:

The dietary polyphenol resveratrol has been shown to have chemopreventive activity against cardiovascular disease and a variety of cancers in model systems, but it is not clear whether the drug reaches the proposed sites of action in vivo after oral ingestion, especially in humans. In this study, we examined the absorption, bioavailability, and metabolism of 14C-resveratrol after oral and i.v. doses in six human volunteers. The absorption of a dietary relevant 25-mg oral dose was at least 70%, with peak plasma levels of resveratrol and metabolites of 491 ± 90 ng/ml (about 2 μM) and a plasma half-life of 9.2 ± 0.6 h. However, only trace amounts of unchanged resveratrol (<5 ng/ml) could be detected in plasma.

Most of the oral dose was recovered in urine, and liquid chromatography/mass spectrometry analysis identified three metabolic pathways, i.e., sulfate and glucuronic acid conjugation of the phenolic groups and, interestingly, hydrogenation of the aliphatic double bond, the latter likely produced by the intestinal microflora. Extremely rapid sulfate conjugation by the intestine/liver appears to be the rate-limiting step in resveratrol's bioavailability. Although the systemic bioavailability of resveratrol is very low, accumulation of resveratrol in epithelial cells along the aerodigestive tract and potentially active resveratrol metabolites may still produce cancer-preventive and other effects.

Resveratrol is a dietary antioxidant polyphenol, found in grapes, red wine, and peanuts, that has been strongly indicated to have protective effects against cardiovascular disease and, in particular, cancer, including all stages of carcinogenesis (Jang et al., 1997; Bhat and Pezzuto, 2002; Pervaiz, 2003). Resveratrol is capable of inhibiting the transcriptional activation of the carcinogen-activating enzyme CYP1A1, thus preventing cancer at the initiation stage. Resveratrol also has the ability to inhibit the promotion of growth of preneoplastic lesions by effects on multiple signaling systems, most recently including activation of the de novo ceramide synthesis pathway (Scarlatti et al., 2003). Thus, based on in vitro studies, resveratrol can inhibit cell proliferation, induce apoptosis, and block cell cycle progression in numerous types of human cancer cell lines, such as those of the colon, skin, breast, lung, prostate, and liver, as well as pancreas. Attempts to extend such in vitro findings to in vivo animal studies, using chemically induced carcinogenesis models, have resulted in a few studies in which effectiveness was shown at modest oral doses of resveratrol. This includes effects on tumors of the colon (Tessitore et al., 2000) and esophagus (Li et al., 2002), i.e., tissues immediately accessible to orally administered drug, and, surprisingly, the mammary glands (Banerjee et al., 2002). The validity of these observations was the focus of a recent review article (Gescher and Steward, 2003). Corresponding in vivo studies of the effects of resveratrol on the cardiovascular system are lacking. It is not known whether dietary resveratrol in vivo will reach the multiple proposed sites of actions beyond the gastrointestinal tract. Although numerous attempts have been made to determine the bioavailability of resveratrol, we have only limited information on this subject in humans (Gescher and Steward, 2003; Goldberg et al., 2003).

To address this question, 14C-labeled resveratrol (Fig. 1) was administered both orally and intravenously to normal, healthy volunteers, greatly facilitating estimates of the extent of the oral dose absorbed, the bioavailability of unchanged drug, and the drug’s metabolic fate. Resveratrol demonstrated high oral absorption but rapid and extensive metabolism, as determined by LC/MS, resulting in only trace amounts of unchanged resveratrol in the systemic circulation. Localized accumulation of resveratrol in epithelial cells along the aerodigestive tract and potentially active resveratrol metabolites may still produce cardiovascular and cancer-preventive effects.

Materials and Methods

Subjects and Study Design. Six healthy subjects (23–34 years; 75–109 kg) participated in the study; three subjects were female; one was black and five were white. Written informed consent was obtained and the study was approved by the Institutional Review Board for Human Research. The oral and intravenous radiation doses were estimated to be about 1% of the annual whole-body background radiation in the United States. All subjects were studied in the Clinical Research Unit. The diet, both during and for 4 days before the study, was low in polyphenols, and grape and peanut products were excluded. Oral (six subjects) and intravenous (five subjects) resveratrol doses, at least a week apart, were administered in the morning, after an overnight fast;

ABBREVIATIONS: LC/MS, liquid chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; amu, atomic mass unit(s); SULT, sulfotransferase; AUC, area under the curve.
breakfast was served 3 h later. Serial blood samples drawn over 0 to 72 h after the dose were centrifuged to separate plasma. Six 12-h urine samples were collected with sodium bisulfite as preservative. Stools were collected for 72 h and homogenized separately with 1 M acetic acid, and aliquots of all samples were stored at −20°C.

**14C-Resveratrol Doses.** Whereas the daily dietary intake of resveratrol is difficult to estimate (low milligram levels), resveratrol is also supplied by the health food industry as a dietary supplement in 20- to 50-mg doses. We therefore selected 25 mg as a representative oral dose. To be able to determine the absolute absorption and bioavailability, we also administered a small intravenous dose of 0.2 mg. The oral 14C-resveratrol dose consisted of 25 mg (110 μmol) of resveratrol and 50 mg of ascorbic acid (Sigma-Aldrich, St. Louis, MO); dissolved in 1 ml of ethanol, with 50 μCi of 14C-resveratrol (61.3 mCi/mmol; National Cancer Institute Radiochemical Repository at Chemsyn Science Laboratories, Lenexa, KS; radiochemical purity ≥95% by HPLC) added in 50 μl of DMSO. Immediately before administration, 19 ml of Simple Syrup (Humco, Texarkana, TX) was added with vigorous shaking to form a slightly cloudy solution. The oral dose was followed by 250 ml of water.

For the intravenous doses, 1.5 mg of 14C-resveratrol (400 μCi) was dissolved in 1.5 ml of 100% ethanol and sterilized by filtration. The solution was tested for sterility and pyrogens and stored at −80°C. Immediately before administration, 0.2 ml of this solution was added to sterile saline, and 10 ml (0.2 mg, 0.8 μmol 14C-resveratrol, 50 μCi) was infused with an equal volume of saline over 10 min. The infusion line was then rapidly flushed with 10 ml of saline.

**Sample Analysis for Total Radioactivity.** Plasma and urine samples (in duplicates) were counted directly after the addition of an equal volume of water and 10 ml of Aquasol-2 scintillation cocktail (PerkinElmer Life and Analytical Sciences, Boston, MA). The radioactivity content in the fecal homogenates was estimated by freeze-drying duplicate 2-g aliquots and extraction (Varin et al., 1987). The extracts were taken to dryness, reconstituted in the mobile phase, and analyzed by HPLC with fraction collection as described (Falany et al., 1995; Ganguly et al., 1995), were incubated with 1 to 100 μM resveratrol and 1 μM 14C-resveratrol, the recovery of added radioactivity in the methanol extract was 89 ± 3%.

Aliquots of plasma extracts were also incubated with alysesulfatase or β-glucuronidase before analysis as follows. For alysesulfatase incubation, dried methanol extracts were redissolved in pH 7.4 Tris buffer. To one half was added 10 μl of alysesulfatase (Aerobacter aerogenes; Sigma-Aldrich). Both samples were incubated overnight at 37°C. After the addition of an equal volume of methanol and centrifugation, the supernatants were analyzed by HPLC with UV detection and fraction collection for determination of radioactivity. For β-glucuronidase incubation, other aliquots of the methanol extracts were redissolved in pH 4.7 acetate buffer and incubated overnight with or without 10 mg of beef liver β-glucuronidase (Sigma-Aldrich) and analyzed as above.

**Calculations.** The areas under the plasma concentration-time curves (AUCs) were calculated by the trapezoidal rule to the last time point, 72 h. The urine half-lives were calculated by least-squares linear regression of the percentage of the dose recovered in each 12-h collection versus the midpoint of the collection interval.

**Sulfate Conjugation of Resveratrol by Sulfotransferase (SULT) Isoforms.** Since resveratrol sulfate standards were not available, reference HPLC standards were produced using recombinant sulfotransferases. SULT1A1 (Pan-Vera Corp., Madison, WI), SULT1A3, and SULT1E, the two latter enzymes being expressed in Aerobacter aerogenes, were expressed in E. coli. 1-ml plasma samples were spiked with 14C-resveratrol, the recovery of added radioactivity in the methanol extract was 58 ± 7%.

**Isolation of Metabolites from Urine.** The 0- to 12-h urine samples were analyzed for resveratrol and metabolites after solid-phase extraction. Urine samples and their fractions were dried to near dryness under nitrogen and resuspended in 1 ml of a C18 mobile phase, and analyzed by HPLC with fraction collection as described (Falany et al., 1995; Ganguly et al., 1995), were incubated with 1 to 100 μM resveratrol and 1 μM 14C-resveratrol, the recovery of added radioactivity in the methanol extract was 89 ± 3%.

**Sulfate Conjugation of Resveratrol by Sulfotransferase (SULT) Isoforms.** Since resveratrol sulfate standards were not available, reference HPLC standards were produced using recombinant sulfotransferases. SULT1A1 (Pan-Vera Corp., Madison, WI), SULT1A3, and SULT1E, the two latter enzymes being expressed in Aerobacter aerogenes, were expressed in E. coli. 1-ml plasma samples were spiked with 14C-resveratrol, the recovery of added radioactivity in the methanol extract was 58 ± 7%.

**Isolation of Metabolites from Urine.** The 0- to 12-h urine samples were analyzed for resveratrol and metabolites after solid-phase extraction. Urine samples were spiked with 14C-resveratrol (110 μmol) in six human subjects, an early peak plasma resveratrol equivalent concentration, i.e., total radioactivity, of 491 ± 90 ng/ml (mean ± S.E.M.), or about 2 μM, was reached at about 1 h after the dose (Fig. 2). At 6 h after the dose, there was a second peak in all subjects with a mean concentration of 290 ± 68 ng/ml (1.3 μM). The plasma concentrations then declined exponentially. After an i.v. dose of 0.2 mg of 14C-resveratrol (0.8 μmol) in five subjects, there was a rapid fall of the plasma concentrations of total radioactivity over the first hour after the bolus injection, indicating the distribution phase. There was no indication of a second peak in any of the subjects. The plasma concentrations then fell in parallel with those after the oral dose for the remainder of the 72-h study period.

**Results**

**Total Radioactivity. Plasma.** After an oral dose of 25 mg of 14C-resveratrol (110 μmol) in six human subjects, an early peak plasma resveratrol equivalent concentration, i.e., total radioactivity, of 491 ± 90 ng/ml (mean ± S.E.M.), or about 2 μM, was reached at about 1 h after the dose (Fig. 2). At 6 h after the dose, there was a second peak in all subjects with a mean concentration of 290 ± 68 ng/ml (1.3 μM). The plasma concentrations then declined exponentially. After an i.v. dose of 0.2 mg of 14C-resveratrol (0.8 μmol) in five subjects, there was a rapid fall of the plasma concentrations of total radioactivity over the first hour after the bolus injection, indicating the distribution phase. There was no indication of a second peak in any of the subjects. The plasma concentrations then fell in parallel with those after the oral dose for the remainder of the 72-h study period.

The terminal elimination half-lives ranged from 7 to 14 h after all doses (Table 1). The interindividual variability in AUC values for both the oral and i.v. doses was surprisingly small, i.e., about 2-fold
concentrations for the initial 12 h in a linear plot. Most error bars are within the symbols. The inset depicts the oral dose concentrations for the initial 12 h in a linear plot.

**FIG. 2.** Plasma concentration-time curves for total radioactivity after oral 25-mg (110 μmol) (○) and intravenous 0.2-mg (0.8 μmol) (□) [14C]-resveratrol doses in human subjects. Mean values ± S.E.M. from six oral doses and five i.v. doses are shown. Most error bars are within the symbols. The inset depicts the oral dose concentrations for the initial 12 h in a linear plot.

**TABLE 1**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Oral Dose</th>
<th>i.v. Dose</th>
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<tbody>
<tr>
<td></td>
<td>t(_{1/2})</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>9530</td>
</tr>
<tr>
<td>6</td>
<td>9.3</td>
<td>6000</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>9.2 ± 0.6</td>
<td>6240 ± 680</td>
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(Table 1), with the highest values in the smallest subject. It should be emphasized that the plasma levels and derived AUC and t\(_{1/2}\) values represent a mixture of chemical species, which may vary between individuals and between routes of administration.

**Urine and Feces.** Most of the radioactivity after the oral doses was recovered in urine (53.4–84.9%). The recovery in feces was highly variable (0.3–38.1%) (Table 2). After the i.v. dose, the recoveries in urine were 42.3 to 83.2% of the dose with 0.6 to 22.7% found in the feces. Thus, the overall recoveries in urine and feces were 70.5 to 97.6% after the oral and 53.5 to 91.2% after the intravenous dose. The elimination half-lives in urine were similar to those in plasma, 6.5 to 14.9 h after oral doses and 7.5 to 18.8 h after i.v. doses (data not shown).

**Resveratrol and Resveratrol Metabolites in Urine.** For structure identification of resveratrol metabolites by LC/MS, we could not use radioactive doses of resveratrol. Also, to optimize the conditions for detection, a larger unlabeled dose, 100 mg, was given orally to one of the subjects. After solid-phase extraction of the 0- to 12-h urine, LC/MS/UV analysis, using a 0.1% acetic acid/methanol gradient as the mobile phase, produced the tracings in Fig. 3. Figure 3A, focusing on the molecular ions characteristic of resveratrol metabolites, detected five major metabolites, as indicated in the figure. The mass spectra of these metabolites were remarkably simple, with very intense [M – H]\(^{-1}\) ions. Metabolite 1 (M1) is a resveratrol monoglu-
m/z 227. In analogy with M3, M5 had an [M − H]⁻ ion of 2 amu higher, i.e., 309, than M4 and also fragmented by losing 80 amu to form the product ion of m/z 229. Trace amounts of unchanged resveratrol, [M − H]⁻ of m/z 227, could be detected when samples were recorded by multiple reaction monitoring LC/MS.

M1, M2, and M4 had clear UV absorption peaks at 305 nm (Fig. 3B), whereas M3 and M5 did not, suggesting that M3 and M5 most likely had lost the conjugating double bond between the two aromatic rings. The relatively poor HPLC properties of the two sulfate conjugates M4 and M5, using acetic acid as modifier in the mobile phase, is consistent with a previous observation (Kaldas et al., 2003). The LC/MS evidence of dihydroresveratrol in Fig. 3 prompted synthesis of this potential metabolite by platinum-catalyzed hydrogenation of resveratrol, as described under Materials and Methods. Its structure was confirmed by NMR.

Because no synthetic standards were available, the LC/MS data only provided qualitative, rather than quantitative, information. Attempts to quantify the metabolites in Fig. 3 by radioactivity after the 25-mg oral as well as the 0.2-mg i.v. doses using the mobile phase in Fig. 3 were unsuccessful due to the severe tailing of the sulfate conjugates M4 and M5. This was greatly improved by changing the mobile phase to contain 0.3% trifluoroacetic acid. However, now the glucuronides M1, M2, and M3 coeluted, as did the sulfate conjugates M4 and M5.

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Because this metabolite disappeared after incubation with aryl sulfatase, with a concomitant increase in the resveratrol peak, it can be concluded that this peak is a sulfate conjugate. It also had the same retention time as the urinary sulfate conjugates M4/M5 in Fig. 4. When examining the 30-min samples from the same subjects, the plasma from subjects 1 and 2 had no resveratrol, whereas in subject 3 there was a small amount of resveratrol left, but mostly the sulfate conjugate, M4/M5. In samples obtained beyond 30 min, there was no unchanged resveratrol detected in any of the subjects. Similar to the 30-min plasma sample after the i.v. dose, 2 h after the oral dose in one subject there was a large M4/M5 peak in plasma with no resveratrol. After aryl sulfatase hydrolysis, this peak disappeared with the appearance of resveratrol, with an estimated concentration of 124 ng/ml.

Discussion

This is the first comprehensive study, in humans, of the disposition of the natural product resveratrol, which is widely claimed to prevent cardiovascular disease and, in particular, cancer. The study used both oral and i.v. doses to assess the extent of absorption as well as the bioavailability of unchanged drug. It also used radiolabeled resveratrol to maximize our observations on both parent resveratrol and its metabolic products in plasma as well as urine and feces.

Based on the urinary excretion data, the absorption of resveratrol appears to be at least 70%. This finding may also be inferred from the plasma total AUC data, if resveratrol is assumed to be metabolized similarly after oral and i.v. doses. This absorption rate is unusually high for a dietary polyphenol. However, in great contrast, the oral bioavailability of unchanged resveratrol is almost zero due to rapid and extensive metabolism, resulting in little unchanged resveratrol in the systemic circulation, but a fairly high (maximum 2 μM) concentration of resveratrol metabolites after the 25-mg oral dose. The observation that the oral bioavailability for resveratrol is negligible agrees well with the findings in a previous preliminary study in four human subjects receiving an oral dose also of 25 mg (no i.v. dose) and using a sensitive gas chromatography/MS methodology (Goldberg et al., 2003). This earlier study also showed about a 2 μM peak plasma concentration of total conjugated metabolites measured indirectly after enzymatic hydrolysis to resveratrol, i.e., complementary to the present study. The good agreement between the two studies regarding the bioavailability of resveratrol emphasizes the use of radioactive doses and HPLC as well as unlabeled doses and gas chromatography/MS as methods of choice in the continuing investigation of the absorption and bioavailability of dietary polyphenols.

The high oral absorption of resveratrol is consistent with findings in the human intestinal Caco-2 cell monolayer (Kaldas et al., 2003). Resveratrol transport in this study was direction-independent and occurred principally by transepithelial diffusion. However, the transport of resveratrol was nonlinear with time, suggesting metabolism to be rate-limiting with respect to bioavailability. This transport study also found extensive sulfate conjugation, in particular, but also glucuronic acid conjugation. Although presystemic metabolism of resveratrol may be important for the oral dose, our observations for the i.v. dose also demonstrate highly efficient systemic metabolism. Based on a comparison of total plasma AUC values adjusted for the doses used for the oral and i.v. administration, there does not appear to be an important dose disproportionality in the metabolism of resveratrol. The second peak of plasma total radioactivity at 6 h after the oral dose may be due to enteric recirculation of conjugated metabolites by reabsorption after intestinal hydrolysis.

For the metabolic studies of resveratrol, the use of radioactive doses and LC and LC/MS techniques were clearly the methods of choice. The qualitative metabolic fate of resveratrol was well characterized in the present study from the urinary excretion data. Thus, two isomeric glucuronic acid conjugates and one sulfate conjugate were positively identified, although the exact positions of these conjugations in the resveratrol molecule were not established. This is similar to previous recent work carried out with human liver microsomes and hepatocytes (Yu et al., 2002). There was no evidence of enzymatic oxidation of resveratrol in our human study. The molecule already has three phenolic hydroxyl groups. Interestingly, strong evidence for hydroxylation, rather than oxidation, of the aliphatic double bond was obtained, with this reduced metabolite excreted both in a glucuronic acid and a sulfate conjugate form. This identification was supported by its chemical synthesis and characterization by NMR spectrometry. Importantly, this novel metabolite totally lost its UV absorption properties at the 306-nm wavelength characteristic of resveratrol. Thus, it was only by MS that it could be recognized, both as a glucuronic acid and as a sulfate conjugate, together constituting what appears to be an important fraction of the dose. The origin of this dihydroresveratrol metabolite can only be speculated on. Alkenes are in general subject to cytochrome P450-mediated oxidations. Reductions of such molecules may be mediated by the intestinal microflora (Parkinson, 2001).

The distinct effect of acetic acid versus trifluoroacetic acid as the modifier in the mobile phase used for HPLC has no simple explanation. Thus, whereas acetic acid gave superior separation efficiency between resveratrol metabolites, the peak symmetry of the sulfate conjugates (Fig. 3) was very poor, as previously noted (Kaldas et al., 2003), making this mobile phase unsuitable for measurements of the sulfate conjugates after the 25-mg oral dose as well as after the i.v. dose. Conversely, trifluoroacetic acid, while giving excellent peak symmetry, gave very poor resolution of individual glucuronic acid as well as sulfate conjugates (Fig. 4). Also, whereas acetic acid in the mobile phase markedly retained the sulfate conjugates to elute after resveratrol itself (Fig. 3), with trifluoroacetic acid, the sulfate conjugates eluted before resveratrol (Fig. 4). A similar pattern was observed for sulfate conjugates of the flavonoid chrysin (Galijatovic et al., 1999; Walle et al., 1999). The poor chromatographic behavior of sulfate conjugates is most likely the reason why very few observations exist for these conjugates as compared with glucuronic acid conjugates of many polyphenols.

The oral bioavailability of unchanged resveratrol established from our plasma data were close to zero. A maximum concentration estimated in plasma of total metabolites was about 2 μM, as measured by total radioactivity. Our attempts to quantify in plasma the individual metabolites found in urine were largely unsuccessful due to their low systemic concentrations and apparently high plasma binding, as well as limited stability. However, one metabolite, i.e., the sulfate conjugate M4/M5, was clearly detectable in plasma samples within 2 h after either i.v. or oral doses. The extremely rapid formation of this metabolite (Fig. 5) indicates that sulfation might be the main limiting factor to the bioavailability of resveratrol. In preliminary experiments using recombinant sulfotransferases, we could show that resveratrol was a substrate for SULT1A1, SULT1A3, and SULT1E1, all isoforms expressed in the intestine.

A large number of studies of the disposition of resveratrol in laboratory animals have been conducted, as recently reviewed (Geschler and Steward, 2003), and as seen in two recent studies (Yu et al., 2002; Meng et al., 2004). In general, the doses of resveratrol have been higher in animals than in humans. However, as in humans, the oral bioavailability in animals seems to be low and the metabolism involves both glucuronidation and sulfation. Although studies in some laboratory animals suggest that resveratrol may have sufficient bioavailability to produce chemopreventive effects (Tessitore et al., 2000; Banerjee et al., 2002; Li et al., 2002), it appears clear that this
may not be the case in humans, unless other factors are taken into consideration. One such very important factor could be the site(s) of chemopreventive action. A number of studies have been focusing on colon cancer. We have already demonstrated a very high accumulation of resveratrol in the intestinal epithelial Caco-2 cells in comparison with the incubation buffer, emphasizing that the enterocyte/colonocyte could be a major biological target site for this dietary preventive compound (Kaldas et al., 2003). This may also be true with other epithelial cells along the aerodigestive tract, such as oral and esophageal cells (Walle, 2004). Thus, an uptake study in the human oral epithelial SCC-9 cells demonstrated extremely rapid and extensive uptake of resveratrol in this cell type also (T. Walle and A. Browning, unpublished data). The rapid uptake by these cells would make them able to capture resveratrol during the relatively short transit time of oral ingestion. Also, preliminary experiments show antiproliferative effects of dihydroresveratrol in the same cell line, with slightly less potency than resveratrol. Other biological properties of dihydroresveratrol have also been examined (Kageura et al., 2001; Stivala et al., 2001). Since the dihydroresveratrol metabolite most likely is formed in the colon, it might contribute to chemopreventive effects at that site.

In contrast, based on the bioavailability observations, it would seem that target sites such as the breast and prostate, where some interesting resveratrol cell culture research has been done, would be excluded from cancer-preventive actions of resveratrol, unless biologically active metabolites are invoked. Resveratrol sulfate, a critically important metabolite, is formed in the colon, it might contribute to chemopreventive effects at that site.

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References
