Comparison of the effects of the chemopreventive agent resveratrol and its synthetic analogtrans 3,4,5,4?-tetramethoxystilbene (DMU-212) on adenoma development in the ApcMin+ mouse...

ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · JUNE 2005

Impact Factor: 5.09 · DOI: 10.1002/ijc.20884 · Source: PubMed

Int. J. Cancer: **115,** 194–201 (2005)

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

Stewart Sale[1], Richard G. Tunstall[1], Ketan C. Ruparelia[2], Gerry A. Potter[2], William P. Steward[1] and Andreas J. Gescher[1]*

[1]*Cancer Biomarkers and Prevention Group, Department of Cancer Studies, University of Leicester, Leicester, United Kingdom*
[2]*Cancer Drug Discovery Group, School of Pharmacy, DeMontfort University, Leicester, United Kingdom*

Naturally occurring molecules with putative cancer chemopreventive properties such as the phytoalexin resveratrol (3,5,4′-trihydroxystilbene) are lead molecules that guide the design of novel agents with improved pharmacologic properties. The synthetic resveratrol analog 3,4,5,4′-tetramethoxystilbene (DMU-212) has been shown to possess stronger antiproliferative properties in human colon cancer cells than resveratrol. We tested the hypothesis that DMU-212 is also a more potent inhibitor of adenoma development in the Apc^{Min+} mouse, a model of human intestinal carcinogenesis. Apc^{Min+} mice received either stilbene derivative with the diet (0.2%), and adenomas were counted after experiments were terminated. Resveratrol and DMU-212 decreased adenoma load by 27% and 24%, respectively, compared to untreated controls. Cyclooxygenase (COX) enzymes are important mechanistic targets of resveratrol, and we investigated whether DMU-212 interferes with the expression and activity of COX in human colon cells. Incubation of HCA-7 cancer cells for 24–96 hr with either stilbene derivative (1–50 μM) decreased prostaglandin E-2 (PGE-2) production, but only resveratrol decreased COX-2 protein expression. In mice, which received either stilbene derivative (0.2%) for 3 weeks with their diet, PGE-2 levels in the intestinal mucosa were reduced by between 45% and 62% compared to mice on control diet. While resveratrol inhibited enzyme activity in purified COX preparations, DMU-212 failed to do so. The PGE-2 decrease seen with DMU-212 in cells and *in vivo* is probably mediated via its metabolites. The results suggest that alteration of the resveratrol molecule to generate DMU-212 does not abrogate its ability to decrease adenoma number in Apc^{Min+} mice or to interfere with PGE-2 generation in cells.

Key words: Apc^{Min+} mouse; analog development; chemoprevention; resveratrol

The cancer chemopreventive activity of many plant-derived mixtures has been pinpointed to defined molecular entities contained within them. For example, the cancer chemopreventive activity of green tea, leafy green vegetables and red grapes has been associated with epigallocatechin gallate, indole-3-carbinol and the polyphenolic phytoalexin resveratrol (*trans* 3,4′,5-trihydroxystilbene; for structure, see Fig. 1), respectively.[1] It is not known whether these phytomolecules possess optimal pharmacologic properties, or if analogs with superior pharmacologic profiles could be chemically synthesized. From a mechanistic standpoint, cancer chemopreventive phytochemicals are multitargeted molecules, and the rational development of analogs is confounded by the fact that suitable biologic systems to test for efficacy are not easy to define. Therefore, it is not surprising that knowledge of the relationship between the structure of such agents and their cancer chemopreventive activity, while highly desirable, is rather scarce. Mindful of these considerations, we compare in this article the potential cancer chemopreventive efficacy of the synthetic resveratrol analog 3,4,5,4′-tetramethoxystilbene (DMU-212; Fig. 1) with that of the naturally occurring lead molecule. Pharmacokinetic and mechanistic findings reported recently[2] suggest that DMU-212 possesses pharmacologic properties superior to those of resveratrol.

Resveratrol is generated in response to environmental stress or pathogenic attack in grapes, mulberries, cranberries, peanuts and plants of the *Cassia quinquangulata* family. It inhibits diverse cellular events associated with the initiation, promotion and progression of cancer,[3] proliferation of a variety of cancer cell lines,[4] formation of preneoplastic lesions in the dimethylbenzathracene-induced mouse mammary organ culture model[5] and benzo(*a*)pyrene-induced transformation of rat tracheal epithelial cells.[3] Resveratrol possesses antioxidant, antiinflammatory, antiangiogenic, antimutagenic, kinase-inhibitory and both pro- and antiestrogenic properties.[3,6–9] An important mechanistic feature of resveratrol thought to contribute to its cancer chemopreventive activity is its ability to induce apoptosis.[10,11] In animal studies, resveratrol interfered with the development of carcinogen-induced mammary and colorectal neoplasia[5,12] and decreased the number of adenomas in the small intestine and colon of Apc$^{Min/+}$ mice,[13] although the efficacy of resveratrol in this model was not confirmed in a more recent study.[14] Apc^{Min+} mice harbor a germline mutation in the adenomatous polyposis coli (*Apc*) gene and are characterized by inactivation of *Apc*, nuclear accumulation of β-catenin and enhanced expression of specific genes activated by T-cell factor (TCF)/β-catenin signaling. They are therefore a model of human intestinal carcinogenesis that is related to inactivated *Apc*.

Among the biochemical events engaged by resveratrol, which may mediate induction of apoptosis, is interference with the activity of cyclooxygenase (COX) by enzyme inhibition and/or down-regulation.[15–23] These findings characterize COX enzymes as potentially important targets of stilbenes. COX-1 and COX-2 catalyze the conversion of arachidonic acid to protumorigenic eicasonoids, such as prostaglandin E-2 (PGE-2), which are involved in the maintenance of the malignant phenotype.[24] While COX-1 is constitutively expressed ubiquitously, COX-2 expression is upregulated during inflammation, in 80–85% of human adenocarcinomas and colonic tumors,[25,26] and also in Apc^{Min+} mouse adenomas.[27] Abrogation of intestinal prostaglandin production via inhibition of COX catalysis is thought to contribute substantially to the Apc^{Min+} adenoma-suppressing activity of the nonsteroidal antiinflammatory drugs sulindac,[28] piroxicam[29,30] and the COX-2 inhibitor celecoxib.[31]

The trihydroxystilbene scaffold of resveratrol has been the subject of synthetic manipulations by medicinal chemists with the aim of generating novel resveratrol analogs. Structural alterations have been aimed at optimization of cytochrome P450 enzyme-inhibitory and antimutagenic potencies,[32,33] antioxidant activity,[34] apoptosis-inducing and growth-inhibitory activity[32,34,35] and ability to inhibit cell transformation.[36] These chemical synthetic attempts have predominantly been concerned with the introduction into the trihydroxystilbene framework of additional hydroxy moieties and with various degrees of phenol group methylation. 3,4,5,4′-tetrahydroxystilbene, resveratrol with an additional

Grant sponsor: the U.K. Medical Research Council; Grant sponsor: the Association for International Cancer Research.
***Correspondence to:** Department of Cancer Studies, University of Leicester, RKCSB, Leicester LE2 7LX, United Kingdom.
Fax: +44-1162231855. E-mail: ag15@le.ac.uk
Received 15 October 2004; Accepted after revision 18 November 2004
DOI 10.1002/ijc.20884
Published online 1 February 2005 in Wiley InterScience (www.interscience.wiley.com).

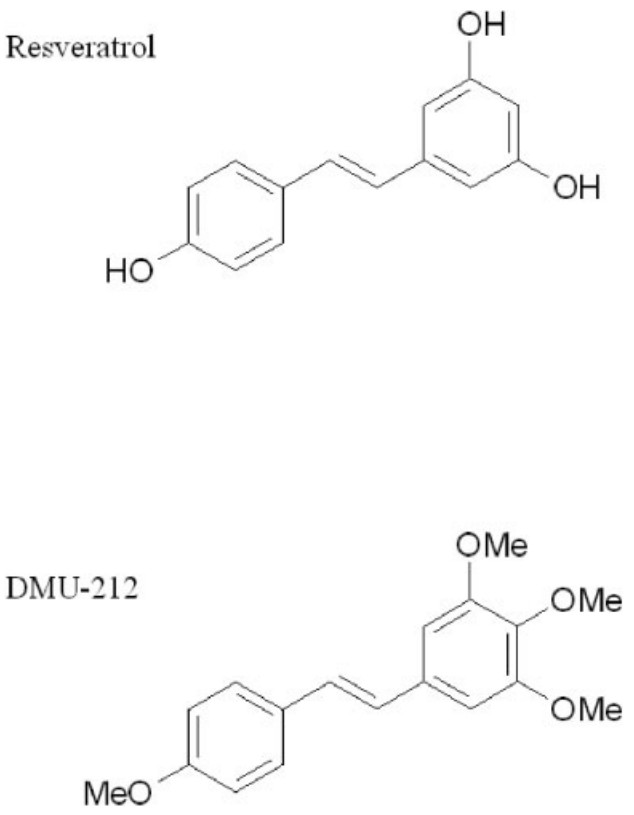

FIGURE 1 – Structures of resveratrol and DMU-212.

hydroxy moiety, and its O-methylated analog DMU-212 were found to interfere preferentially with proliferation and survival of transformed human lung-derived cells, with much lower growth-inhibitory and apoptogenic properties against their untransformed counterparts.[34] In contrast, resveratrol did not possess this discriminatory potential. Recently, DMU-212 was reported to be 4 times more potent than resveratrol as an inhibitor of the growth of human-derived HCA-7 colon cancer cells, with respective IC_{50} values of 6 and 26 μM.[2] In the light of these differences between DMU-212 and resveratrol, we wished to investigate whether the superior growth-inhibitory properties of DMU-212 vis-à-vis resveratrol in colonic cells are predictive of a higher ability to retard the formation of adenomas in the Apc^{Min+} mouse model, and whether the difference in cell growth-inhibitory potential translates into differential capability to interfere with COX enzymes. To that end, both agents were compared in terms of ability to inhibit adenoma development in the Apc^{Min+} mouse and to modulate COX. Their effect on COX was studied in 3 different systems: COX-2 protein expression in human colon-derived cells, COX activity as reflected by PGE-2 levels in colon cells and in the intestinal mucosa of mice, as well as COX activity in purified enzyme preparations. Overall, the study was designed to help rationalize the choice of resveratrol analogs for development as cancer chemopreventive agents.

Material and methods

Chemicals and reagents

The stilbene derivatives used in this study were the *trans* isomers. Resveratrol was obtained from Changchun Kingherb International (Changchun, China). DMU-212 was synthesized by Wittig olefination involving reaction of 4-methoxybenzyl-triphosphonium chloride with 3,4,5-trimethoxybenzaldehyde.[37] This reaction yielded the *cis* and *trans* geometric isomers, which were separated by preparative column chromatograph; the *trans* isomer was purified by recrystallization from ethanol. Purity of the stilbene derivatives was established as at least 99% by high-pressure liquid chromatography (HPLC) analysis.[2] Stilbene derivatives were mixed with AIN93G standard diet (Dyets, Bethlehem, PA), and their uniform distribution in the diet was confirmed by HPLC analysis. All other chemicals were obtained from Sigma-Aldrich (Poole, U.K.) unless otherwise specified.

Animals and treatments

Animal experiments, conducted as stipulated by the Animals (Scientific Procedures) Act 1986 U.K. Home Office Project Licence 40/2496, were approved by the Leicester University Animal Welfare Committee and complied with the UKCCCR guidelines for the welfare of animals in experimental neoplasia.[38] A breeding colony was established using male C57BL/6J $Apc^{Min/+}$ and female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME). The Min genotype of offspring was confirmed by polymerase chain reaction using primers synthesized by the Protein and Nucleic Acid Chemistry Laboratory at Leicester University, and *Hind*III digest of the product, essentially as described by Luongo *et al*.[39] At 4 weeks of age, animals were allocated randomly into 2 groups, control mice that received AIN93G diet and mice in the intervention group that received stilbene derivatives at 0.05% or 0.2% in the diet. Mice had access to food and water *ad libitum*. At 14–18 weeks of age, animals were culled by cardiac exsanguination under terminal halothane anesthesia. The gastrointestinal tract was flushed with phosphate-buffered saline (pH 7.3), removed and opened longitudinally. Size, location and number of adenomas in the small intestine and colon were documented, and adenomas were subsequently microdissected under visual magnification (3×). The experiments were conducted with 2 separate cohorts of $Apc^{Min/+}$ mice, which differed in terms of number of intestinal or colonic adenomas, which were 106 ± 9 and 9 ± 2 ($n = 10$), respectively, in the first and 55 ± 9 and 4 ± 1, respectively, in the second cohort ($n = 12$). Therefore, the adenoma number in the control group of the intervention study with 0.2% stilbene derivatives was higher (first cohort) than that of the study in which 0.05% stilbene derivatives were investigated (second cohort). Hematocrit values were determined as described previously.[40] In a separate experiment, steady-state levels of stilbene derivatives and intestinal mucosa PGE-2 levels were determined in male C57BL/6J mice (Charles River, Margate, U.K.), which received control diet or diet containing stilbene derivatives at the above concentrations for a period of 3 weeks from 7 weeks of age.

Analysis of resveratrol and DMU-212 in tissues and plasma

Tissues were homogenized (1:1 volume to tissue mass ratio) in Hepes buffer (50 mM; Sigma, Poole, U.K.) using a handheld glass homogenizer. An aliquot (250 μl) of homogenate or of plasma, to which internal standard had been added, was vortexed, followed by addition of acetonitrile (1 ml). After vigorous shaking, mixtures were kept on ice (5–10 min) and centrifuged (2,800*g*, 10 min, 4°C). The supernatants were dried under a stream of nitrogen and reconstituted in mobile phase (100 μl). Concentrations of stilbene derivatives in blood, liver and intestinal mucosa were determined by HPLC as described by Sale *et al*.[2] using a Varian Pro-Star HPLC system (Varian, Crawley, U.K.) with a Pro-Star 230 solvent delivery system, a Pro-Star 310 UV-visible or a Pro-Star 363 fluorescence detector, a Varian 410 autosampler and an Ultracarb C_{18} column (4.6 mm × 250 mm, 5 μm; Phenomenex, Macclesfield, U.K.). Detection of resveratrol and DMU-212 was by UV (325 nm) and fluorescence (335 nm excitation, 395 nm emission), respectively. The extraction efficiencies (in %) for resveratrol and DMU-212 were as follows: from plasma 102 ± 17 and 67 ± 2, respectively; from tissues 86 ± 7 and 78 ± 15, respectively (mean ± SD; $n = 5–7$).

Cell culture

HCA-7 cells, derived from a mucinous adenocarcinoma of the colon,[41] and human colon epithelial cells (HCECs) were provided by Drs. S. Kirkland (Imperial College, London, U.K.) and A. Pfeifer (Nestec Research Center, Lausanne, Switzerland), respectively. Cells from subculture 20 to 30 were seeded in 90 mm Petri dishes (Nunc, Fisher Scientific, Loughborough, U.K.). HCA-7 cell seeding density was dependent on duration of incubation and chosen in such a way that cells were approximately 75% confluent at the time of analysis. Seeding density was as follows ($\times 10^6$): 2 for 24-hr incubation period, 1 for 48-hr and 0.5 for 96-hr. Cells were grown in DMEM medium containing Glutamax I, glucose (4.5 g/l) and 10% (v:v) fetal calf serum (Gibco, Paisley, U.K.). HCECs were cultured in dishes precoated with Vitrogen 100 (10 μl/ml; Collagen, Palo Alto, CA), human fibronectin (2.5 μg/ml; Sigma) and bovine serum albumin (50 μg/ml; Gibco). HCEC seeding density was 2×10^6 per well, achieving approximately 50% confluency at the time of analysis. Cells were incubated with medium containing resveratrol or DMU-212 (1–50 μM), HCA-7 cells for 24–96 hr and HCECs for 1 hr prior to addition of phorbol 12-myristate 13-acetate (PMA; 50 ng/ml), after which cells were incubated for a further 5 hr in the presence of both PMA and stilbene derivatives.

Analysis of COX-2 and PGE-2 levels

Cellular COX-2 protein levels were determined by Western blot analysis as previously described[42] using a polyclonal antibody against COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Equal protein loading and transfer were checked for by probing for α-tubulin (Santa Cruz). Semiquantitation including correction in relation to the respective α-tubulin band was by densitometric analysis using a Gene Gnome densitometer (Syngene Bio Imaging Systems, Frederick, MD). PGE-2 levels in the extracellular medium were determined using a PGE-2 enzyme immunoassay (EIA) kit (R&D Systems, Abingdon, U.K.). PGE-2 levels in the intestinal mucosa were determined essentially as described by the manufacturer of the kit. Mucosal scrapings were homogenized (6 ml 0.1 M Tris buffer, pH 7.5), centrifuged (700*g*, 10 min, 4°C; Hereaus Megafuge EBA 12) and loaded onto previously washed (ethanol and water, 10 ml each) C_{18} reverse-phase columns (Varian). Columns were washed (water, 15% ethanol and hexane, 10 ml each) and eluted (10 ml ethyl acetate). The eluate was evaporated under nitrogen and reconstituted in ethanol (50 μl) and EIA buffer as provided with the kit (200 μl). Results were expressed as amount of PGE-2 per mg cellular protein as determined by the Bradford assay.[43] Intestinal mucosa in control (untreated) mice contained 42 ± 8 ng PGE-2/mg protein (mean ± SD; $n = 5$).

Analysis of COX activity

Inhibition of COX activity by the stilbene derivatives was investigated using a Chemiluminescent Cyclooxygenase Activity Kit (Assay Designs, Ann Arbor, MI). COX-1 (95% pure) and COX-2 protein (70% pure) originated from seminal vesicles and placenta from sheep, respectively (Alexis Biosciences, Nottingham, U.K.). Enzyme activity was measured following the instructions of the manufacturer of the kit. In this assay, the peroxidative activity of COX enzymes is measured following addition of arachidonic acid and a proprietary cosubstrate, which is metabolized to a chemiluminescent product. Enzyme preparations were incubated for 2 hr with stilbene derivatives (0.1 nM to 50 μM), or for validatory purposes with ibuprofen (0.01 μM to 27 mM) or the COX-2-selective inhibitor NS 398 (0.01–90 μM). Light emission, which was measured over 15 sec using a BMG Fluostar Optima luminometer (BMG Labtech, Aylesbury, U.K.), was directly proportional to residual COX activity. In this assay, resveratrol enhanced COX activity at 10 and 100 nM while inhibiting it at micromolar concentrations. DMU-212 at 10 and 50 μM also augmented enzyme activity. In a validatory experiment, flavonoid phenols related to resveratrol (curcumin, tricin and apigenin) at 1–100 nM similarly augmented enzyme activity, but ibuprofen or NS 398 did not. We tentatively explain the increase in apparent COX peroxidase activity at submicromolar concentrations of resveratrol and related flavonoids in this assay with their strong reducing potential, a supposition that was considered feasible by the commercial providers of the kit.

Statistical evaluation

Values were subjected to one-way ANOVA and Bonferroni *post hoc* testing for statistical significance using SPSS (version 11.0) for Microsoft Windows 2000. Statistical significance was suggested by $p < 0.05$.

Results

Effect of resveratrol and DMU-212 on adenoma development in Apc^{Min+} mice

Apc^{Min+} mice received resveratrol or DMU-212 in their diet at 0.05% or 0.2%, which constitute doses of approximately 60 and 240 mg/kg body weight *per diem*. Both stilbene derivatives were well tolerated and failed to affect animal weight. At 0.2%, they decreased numbers of intestinal adenomas, while 0.05% was inefficacious (Fig. 2). In mice, which received 0.2% resveratrol, mean adenoma numbers were 27% lower than those in untreated Apc^{Min+} mice. Polyp reduction was observed in both the small intestine and colon (Fig. 2). The mean number of adenomas in mice that consumed 0.2% DMU-212 was 24% lower than that in controls. Both stilbene derivatives retarded the development of small adenomas (< 1 mm diameter) in the proximal section, and of small- and medium-size adenomas (1–3 mm diameter) in the middle section of the intestine (Fig. 3). At the late stage of adenoma development, Apc^{Min+} mice suffer from internal bleeding; thus, the hematocrit value in untreated mice at termination of the experiment was 14.5% ± 3.6% as compared to 35.3% ± 2.0% in wild-type mice of a similar age. Intervention with stilbene derivatives did not reverse hematocrit depression.

Levels of resveratrol and DMU-212 in murine intestinal mucosa

At the end of the dietary intervention, steady-state levels of agents in tissues and plasma of Apc^{Min+} mice, which had received dietary resveratrol or DMU-212 (0.05% or 0.2%), were analyzed by HPLC. Levels of agent in the small intestine and colon were highly variable and of a similar order of magnitude. The variability is probably related to differences in eating pattern between individual animals. Intestinal resveratrol levels were 8 ± 4 and 36 ± 48 nmol/g tissue at the low and high dietary doses, respectively; the analogous values for DMU-212 were 6 ± 2 and 26 ± 22 nmol/g tissue. In comparison, hepatic concentrations of resveratrol were between 0.2 and 0.3 nmol/g tissue at either dose, and those of DMU-212 were 0.10 ± 0.03 (0.05% dietary dose) and 0.7 ± 0.4 nmol/g (0.2%). Plasma levels of resveratrol were below the detection limit of the assay (0.1 μM), while those of DMU-212 were in the 0.1–1 μM range, thus just measurable.

Effect of resveratrol and DMU-212 on levels and activity of COX-2 in human-derived colon cells

Stilbene derivatives (1–50 μM) were incubated with HCA-7 cells, which overexpress COX-2 constitutively, or with HCECs, in which COX-2 expression was induced by the phorbol ester PMA. At the end of the incubation period, cell lysates were probed for levels of COX-2 protein and cell culture media for PGE-2. COX-2 expression was downregulated in HCA-7 cells following exposure to resveratrol for 48 or 96 hr (Fig. 4*b* and *c*). Such a decrease in protein level was not seen after 24 hr (Fig. 4*a*), suggesting that during this short time span the rate of degradation of preexisting protein was too slow to allow inhibition of COX-2 expression to be observed. Similarly, in PMA-treated HCECs, resveratrol inhibited COX-2 expression with an IC_{50} of between 5 and 10 μM (Fig. 5), consistent with previous results in human mammary and oral epithelial cells.[20] DMU-212 failed to affect COX-2 levels in HCA-7 (Fig. 4) or HCECs (Fig. 5). In contrast, PGE-2 levels in

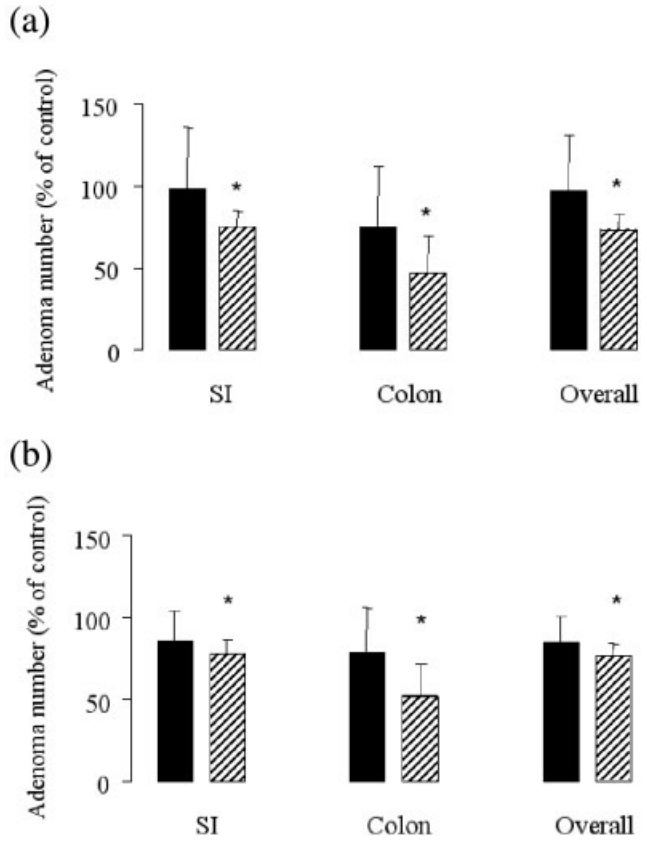

FIGURE 2 – Effect of (*a*) resveratrol and (*b*) DMU-212 in the diet at 0.05 (closed bars) or 0.2% (striped bars) on the number of adenomas in the small intestine (SI) and colon of Apc^{Min+} mice. Animals received dietary stilbene derivatives from week 4 and the experiment was terminated between week 14 and 18. Values are the mean ± SD of 10–12 mice. Experiments were conducted with 2 different colonies of mice; in the study of the effect of 0.2% dietary stilbene derivatives, adenoma numbers in control mice (100% values) were 106 ± 9 in the small intestine and 9 ± 2 in the colon ($n = 10$); in the experiments in which the effect of 0.05% dietary stilbene derivatives was explored, adenoma numbers in control mice were 55 ± 9 in the small intestine and 4 ± 1 in the colon ($n = 12$). Asterisk indicates that adenoma number in treated mice was significantly different from that in untreated ones ($p < 0.05$).

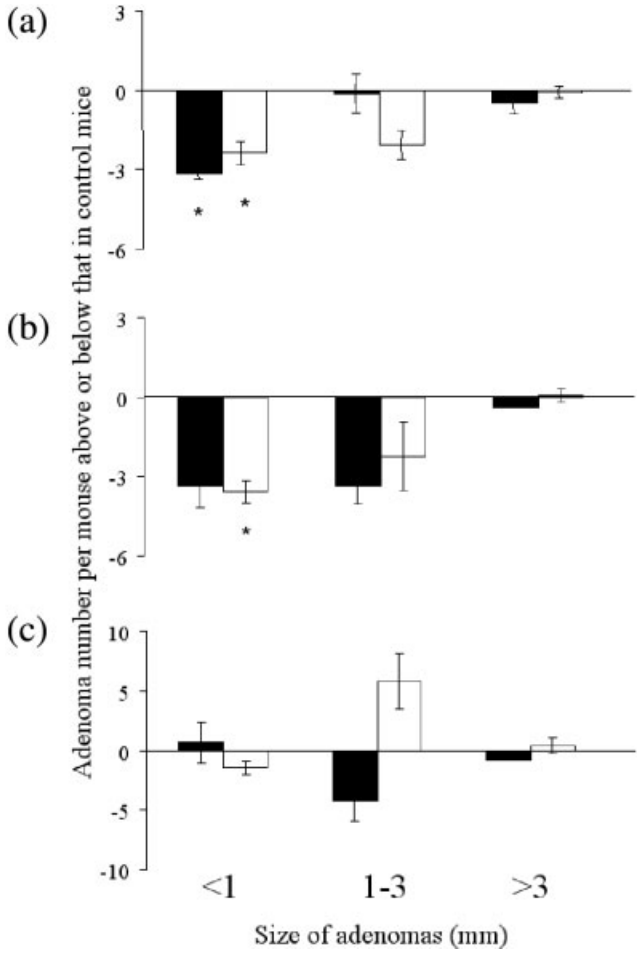

FIGURE 3 – Effect of resveratrol (closed bar) and DMU-212 (open bar) at 0.2% in the diet on multiplicity of small (< 1 mm diameter), medium (1–3 mm), or large (> 3 mm) adenomas in the proximal (*a*), middle (*b*), or distal (*c*) sections of the intestine of Apc^{Min+} mice. Results are expressed as mean number of adenomas over or below mean adenoma numbers in untreated (control) Apc^{Min+}. Animals received dietary stilbene derivatives from week 4 and the experiment was terminated in week 18. The number of adenomas in the proximal, middle and distal region of the small intestine in control mice was as follows: for adenoma size < 1 mm, 2.4 ± 1.5, 4.2 ± 2.4 and 5.6 ± 2.0, respectively; for adenoma size 1–3 mm, 8.2 ± 3.1, 33.2 ± 8.4 and 48.0 ± 8.3, respectively; for adenoma size > 3 mm, 2.6 ± 1.2, 1.3 ± 1.8 and 0.3 ± 0.5, respectively. Numbers of mice per group were between 10 and 12, Asterisk indicates that the number of adenomas was significantly different from that in control animals ($p < 0.05$).

HCA-7 cells were depressed by both resveratrol and DMU-212 at all 3 time points, although the decrease did not reach statistical significance at 96 hr (Fig. 4). PGE-2 levels in HCECs were depressed by both stilbene derivatives at 50 μM only, although the reduction was not statistically significant because of considerable variability between values (Fig. 5).

Effect of resveratrol and DMU-212 on COX activity in a cell-free system

We wished to explore whether the ability of the stilbene derivatives to inhibit PGE-2 generation in HCA-7 and HCECs was the corollary of their enzyme-inhibitory potential. Stilbene derivatives were incubated with isolated COX-1 or COX-2 enzyme preparation, and residual enzyme activity was measured in the presence of arachidonic acid employing a kit in which a cosubstrate of the COX peroxidase reaction generates a chemiluminescent species. In preliminary experiments, a variety of polyphenols, including the stilbenes under study here, at submicromolar concentrations augmented enzyme activity as indicated by this kit. Importantly, however, this augmentation did not mask enzyme-inhibitory potential at higher concentrations. While resveratrol inhibited both COX enzymes with an IC_{50} of between 1 and 10 μM, DMU-212 had no inhibitory activity in this system (Fig. 6). Resveratrol has been reported to be a better inhibitor of COX-1 than of COX-2.[22,23] It interferes with COX-1 peroxidase and inactivates COX-1 cyclooxygenase, while inhibiting only the peroxidase activity of COX-2 via its role as a cosubstrate, without affecting COX-2 cyclooxygenase.[3,22] The enzyme assay kit used here gauged overall COX enzyme activity as reflected by its peroxidase function and thus did not permit detection of the differential susceptibilities of COX-1 and COX-2 cyclooxygenases and peroxidases toward inhibition and inactivation by resveratrol.

Effect of resveratrol and DMU-212 on PGE-2 levels in murine intestine

In the light of the ability of both stilbene derivatives to decrease PGE-2 levels in cells *in vitro* (Figs. 4 and 5), the hypothesis was

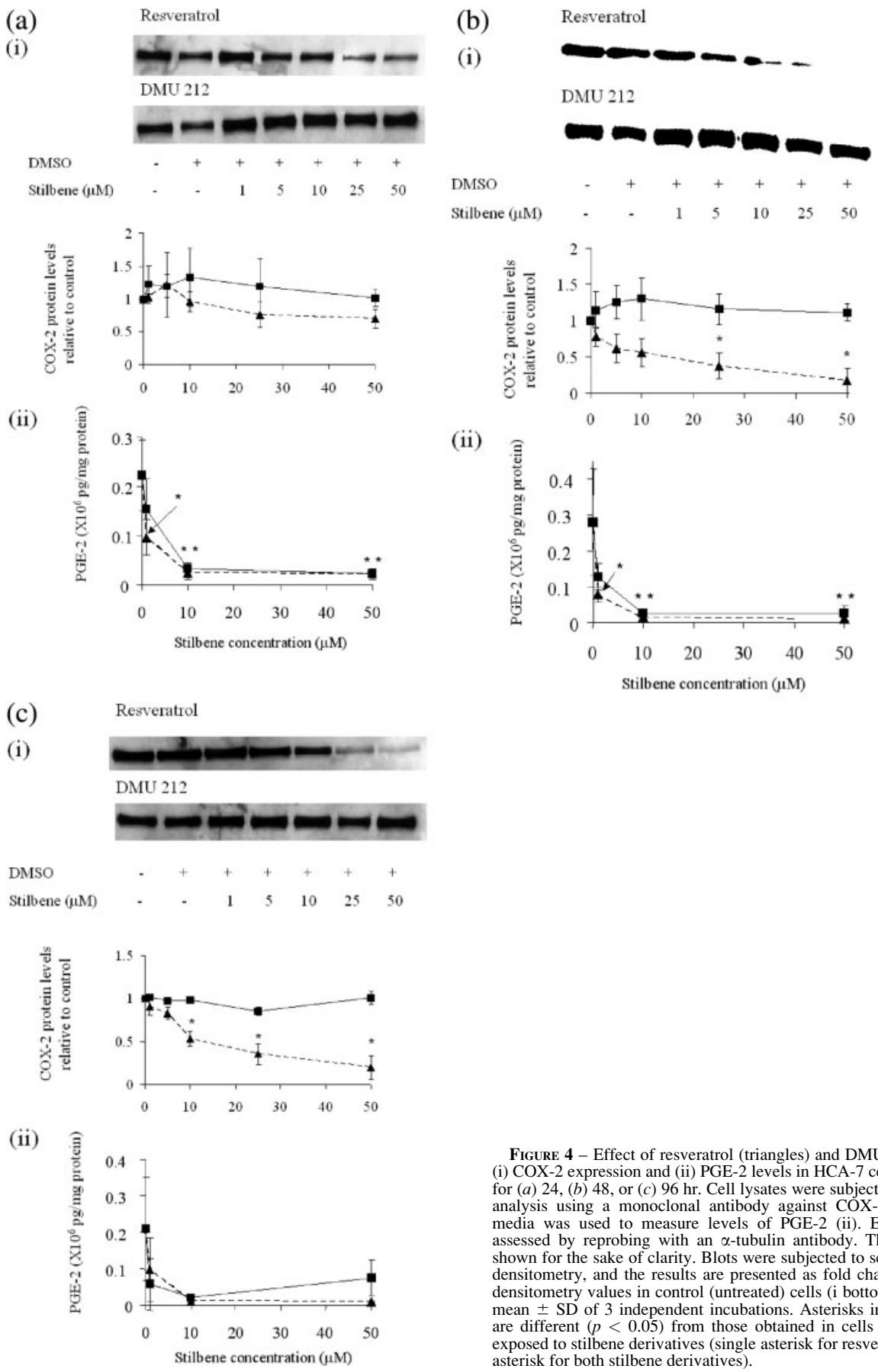

FIGURE 4 – Effect of resveratrol (triangles) and DMU-212 (squares) on (i) COX-2 expression and (ii) PGE-2 levels in HCA-7 cell after incubation for (*a*) 24, (*b*) 48, or (*c*) 96 hr. Cell lysates were subjected to Western blot analysis using a monoclonal antibody against COX-2 (i); cell culture media was used to measure levels of PGE-2 (ii). Equal loading was assessed by reprobing with an α-tubulin antibody. These blots are not shown for the sake of clarity. Blots were subjected to semiquantitation by densitometry, and the results are presented as fold changes compared to densitometry values in control (untreated) cells (i bottom). Values are the mean ± SD of 3 independent incubations. Asterisks indicate that values are different ($p < 0.05$) from those obtained in cells that had not been exposed to stilbene derivatives (single asterisk for resveratrol only; double asterisk for both stilbene derivatives).

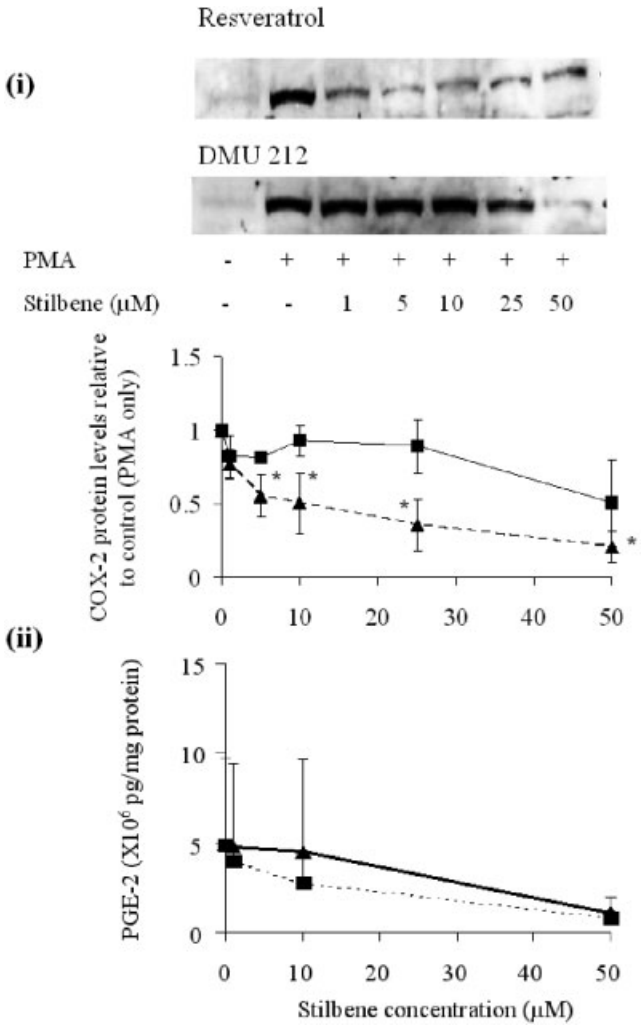

FIGURE 5 – Effect of resveratrol (triangles) and DMU-212 (squares) on (i) COX-2 expression and (ii) PGE-2 levels in HCECs. Cells were exposed to stilbene analogs for 1 hr and to PMA for 5 hr for the measurement of COX-2 or to both for 24 hr for the determination of PGE-2, after which cell lysates were obtained and subjected to (i) Western blot analysis using a monoclonal antibody against COX-2, or (ii) measurement of PGE-2 in the cellular supernatant. Equal loading was assessed by reprobing with an α-tubulin antibody. These blots are not shown for the sake of clarity. Blots were subjected to semiquantitation by densitometry, and the results are presented as fold changes compared to cells treated with PMA only (i bottom). Values are the mean ± SD of 3 independent incubations. Asterisk indicates that value is different from that obtained in cells that had not been exposed to stilbene derivatives ($p < 0.05$).

tested that they also interfere with PGE-2 production by COX-1 catalysis in the rodent intestinal environment *in vivo*. To that end, C57Bl/J6 mice, the Apc^{Min+} background species, received resveratrol or DMU-212 with their diet at 0.05% or 0.2% for 3 weeks, and PGE-2 levels were measured in the intestinal mucosa. Resveratrol reduced intestinal PGE-2 levels significantly by 58% ± 12% and 62% ± 10% ($p < 0.01$), respectively, compared to intestinal mucosa in mice on control diet. DMU-212 also inhibited intestinal PGE-2 production but less effectively than resveratrol, as it reduced levels only at the 0.2% dietary dose, by 45% ± 18% ($p < 0.05$), but not when present in the diet at 0.05%.

Discussion

The results described above can be summarized as follows. One, both stilbene derivatives retarded adenoma formation in the Apc^{Min+} mouse, but resveratrol was somewhat more potent than DMU-212. Two, both resveratrol and DMU-212 interfered with PGE-2 generation in murine intestinal mucosa *in vivo* and in colon-derived cells *in vitro*. Three, resveratrol inhibited COX activity in isolated enzyme preparations, but DMU-212 did not. Four, resveratrol attenuated cellular COX-2 protein expression, while DMU-212 failed to do so. These results allow tentative inferences to be made as to structure-activity relationships among stilbene derivatives related to resveratrol. Alteration of the resveratrol molecule by methylation of the 3 hydroxy moieties and introduction of another methoxy in position 4, which generates DMU-212, abolishes the ability to downregulate COX-2 expression but does not abrogate the potential to inhibit Apc^{Min+} adenoma formation or cellular PGE-2 generation. The precise mechanism of inhibition of PGE-2 production seems to differ between the 2 molecules. DMU-212 has previously been found to inhibit the growth and survival of HCA-7 colon cancer cells more potently than resveratrol,[2] although this superiority does not translate into increased retardation of Apc^{Min+} adenoma formation. For resveratrol to inhibit adenoma development, a dietary concentration of 0.2% (approximately 240 mg/kg) was required, while a quarter

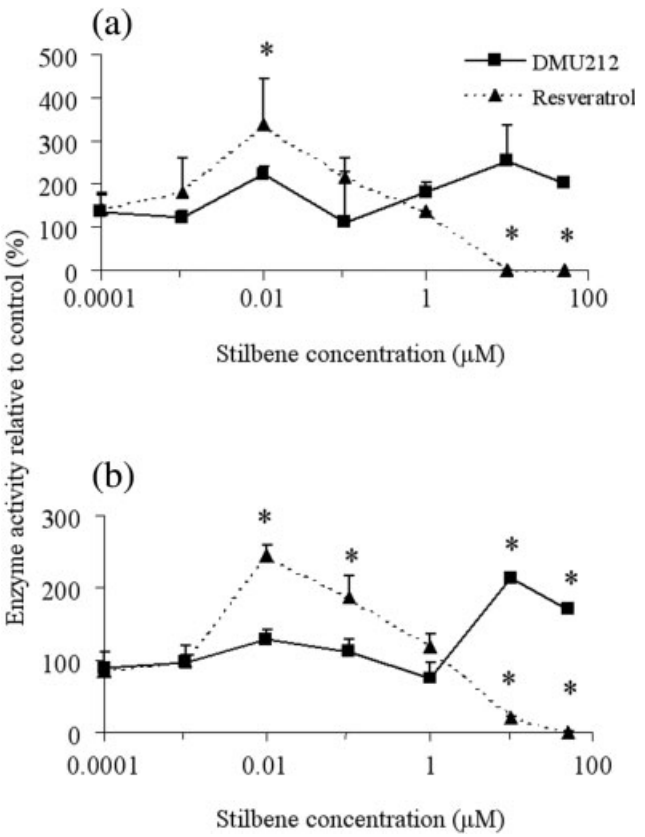

FIGURE 6 – Effect of resveratrol (triangles) and DMU-212 (squares) on activity of (*a*) COX-1 or (*b*) COX-2. Enzyme activity is expressed relative to controls (= 100%) and values are the mean ± SD ($n = 3$–6). Purified COX-1 or COX-2 enzyme preparations were incubated with stilbene derivatives and enzyme activity was measured using a kit in which the COX peroxidase function generates a chemiluminscent product from a cosubstrate. Note that resveratrol at 10 and 100 nM and DMU-212 at 10 and 50 μM augmented enzyme activity. A similar increase in COX enzyme activity was observed with other phenols, *e.g.*, curcumin, at submicromolar concentrations, which may be the corollary of their reducing potential, but not with ibuprofen or NS 398. Asterisk indicates that value differs significantly from control ($p < 0.05$).

of that dose (0.05%) was ineffective. This finding is consistent with a recent report according to which dietary administration of doses of resveratrol (up to 90 mg/kg) failed to affect adenoma number in this model.[14] In contrast, it has been previously suggested that resveratrol at 0.01% in the drinking water (a dose of approximately 12 mg/kg per day) decreased adenoma number by 70%.[13] When this experimental design was repeated in our laboratory, significant reduction in adenoma number was not detected (data not shown).

The ability of resveratrol to interfere with COX activity as described above is broadly consistent with published results in which several different sources of COX enzyme were used. The IC_{50} value for COX inhibition observed here was between 1 and 10 μM, compared with values reported in the literature of 20 μM for COX from ram seminal vesicles,[16] 32 μM for human recombinant COX-2[20] and 0.3–3 μM for PMA- or lipopolysaccharide-induced COX-2 from murine peritoneal macrophages.[17] In contrast, at 439 μM (100 μg/ml), resveratrol apparently failed to inhibit recombinant COX-2 while totally blocking COX-1.[21] Inhibition of COX-1 by resveratrol has recently been shown to be the corollary of mechanism-based enzyme inactivation.[23] The metadihydroxy functionality in the B ring of resveratrol has been suggested to be essential for COX inactivation, which suggests that DMU-212 does not share COX-inactivatory ability with resveratrol. Consistent with this notion, DMU-212 failed to interfere with COX activity in the isolated enzyme preparation, even though it inhibited PGE-2 production in cells and in murine intestine. The most cogent explanation, which rationalizes these apparently paradoxical findings with DMU-212, is that it undergoes metabolic O-demethylation to phenolic cogeners with COX-inhibitory potential, thus exerting enzyme-inhibitory properties in intact metabolically competent cells and in mouse intestine *in vivo*, but not in a cell-free system. The O-demethylated cogeners of DMU-212 4′-hydroxy-3,4,5-trimethoxystilbene, 4-hydroxy-3,4′,5-trimethoxystilbene and 3-hydroxy-4,4′,5-trimethoxystilbene were identified in tissues of mice following oral administration of DMU-212 (240 mg/kg) and also in mouse liver homogenate preparations, which had been incubated with DMU-212.[2] Preliminary experiments suggest that these DMU-212 metabolites inhibit COX potently *in vitro* in the chemiluminescent cyclooxygenase activity kit assay (data not shown), consistent with their potential role as mediaters of DMU-212-induced PGE-2 reduction.

It is tempting to interpret the activities of resveratrol and DMU-212 described here and previously in the light of each other. The superior inhibition of colon cell growth *in vitro* by DMU-212 as compared to resveratrol[2] is not reflected by a corresponding difference in potency as far as inhibition of PGE-2 production in cells *in vitro* is concerned. The similar ability of both stilbene derivatives to retard Apc^{Min+} mouse adenomas seems to mirror their comparable capability to inhibit PGE-2 production *in vitro* and *in vivo*, but not their differential propensity to downregulate COX-2 in cells *in vitro*. So one may tentatively conclude that antiproliferation and COX-2 downregulation are not major mechanistic arbiters of the intestinal adenoma-retarding potency *in vivo* of this type of molecule. In contrast, the ability of the stilbene derivatives to interfere with Apc^{Min+} adenoma development was paralleled by inhibition of PGE-2 production, which might therefore be involved as an antitumor promotional mechanism. This last putative conclusion is consistent with the result of quantitative analysis, according to which intestinal levels of stilbene derivatives achieved in Apc^{Min+} mice following the adenoma-retarding dietary dose of 0.2% were in the 26–36 nmol/g (26–36 μM) range. Based on the study using cells *in vitro* described here, such concentrations would be sufficient to inhibit PGE-2 generation, a notion also borne out by the observed decrease in intestinal PGE-2 levels achieved by both stilbene derivatives in the gut mucosa of C57BL/6J mice. Exploration of any differential potential of the 2 stilbene derivatives to downregulate COX-2 protein expression in Apc^{Min+} adenomas *in vivo* was confounded by the enormous variability between adenomas in COX-2 protein expression, consistent with the previously reported high dependence of COX-2 expression on adenoma size in $Apc^{\Delta 716}$ knockout mice, which are closely related to Apc^{Min+} mice.[44,45] The conclusion as to a potential link between inhibition of COX-catalyzed PGE-2 production and Apc^{Min+} adenoma reduction is based on the seminal finding 10 years ago that genetic inactivation of COX-2 in $Apc^{\Delta 716}$ knockout mice caused a dramatic decrease in gastrointestinal adenoma development.[46] Nevertheless, it is important to realize that in recent years many pharmacologic experiments involving nonsteroidal antiinflammatory drugs have intimated that the degree to which COX inhibition contributes to gastrointestinal cancer chemoprevention in rodents is both difficult to ascertain and is model-dependent.[47] Also, it is important to reiterate that resveratrol is a multitargeted agent,[48] a notion probably also applicable to DMU-212, such that the role which the PGE-2 level-lowering effect of these agents plays in the retardation of Apc^{Min+} adenoma development must be interpreted with caution.

Analog design is a novel approach in cancer chemopreventive agent development aimed at optimizing the cancer chemopreventive potency of naturally occurring agents. This approach is illustrated by the development of SRI 13193[49] and SRI 13668,[50] analogs of epigallocatechin-3 gallate and indole-3-carbinol, respectively, conducted at Stanford Research Institute (SRI) International. The intriguing pharmacologic properties of resveratrol render this molecule a promising candidate for medicinal chemical analog design. While the work described here does not define molecular alterations that improve the potency of the resveratrol molecule in the Apc^{Min+} mouse model, it suggests structural features that can be altered without abrogating its ability to retard Apc^{Min+} mouse adenoma development and to inhibit PGE-2 production. Such knowledge may help guide synthetic strategies to rectify the suboptimal pharmaceutical properties of resveratrol such as its poor systemic bioavailability associated with avid metabolic conjugation.

Acknowledgements

The authors thank Drs. S. Kirkland (Hammersmith Hospital, Imperial College, London, U.K.) and A. Pfeifer (Nestec Research Center, Lausanne, Switzerland) for the provision of HCA-7 and HCECs, respectively.

References

1. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3:768–80.
2. Sale S, Verschoyle RD, Boocock D, Jones DJN, Wilsher N, Potter GA, Farmer PB, Steward WP, Gescher AJ. Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue trans 3, 4,5,4′-tetramethoxystilbene. Br J Cancer 2004;90:736–44.
3. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997;275:218–20.
4. Gusman J, Malonne H, Atassi GA. A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. Carcinogenesis 2001;22:1111–7.
5. Bhat KPL, Lantvit D, Christov K, Mehta RG, Moon RC, Pezzuto JM. Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models. Cancer Res 2001;61:7456–63.
6. Atten MJ, Attar BM, Milson T, Holian O. Resveratrol-induced inactivation of human gastric adenocarcinoma cells through a protein kinase C-mediated mechanism. Biochem Pharmacol 2001;62:1423–32.
7. Haworth RS, Avkiran M. Inhibition of protein kinase D by resveratrol. Biochem Pharmacol 2001;62:1647–51.
8. Uenobe F, Nakamura S, Miyazama M. Antimutagenic effect of resveratrol against Trp-P-1. Mutat Res 1997;373:197–200.
9. Gehm BD, McAndrews JM, Chien PY, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci USA 1997;94:14138–43.

10. Mahyar-Roemer M, Katsen A, Mestres P, Roemer K. Resveratrol induces colon tumor cell apoptosis independently of p53 and preceded by epithelial differentiation, mitochondrial proliferation and membrane potential collapse. Int J Cancer 2001;94:615–22.
11. Mahyar-Roemer M, Kohler H, Roemer K. Role of Bax in reveratrol-induced apoptosis of colorectal carcinoma cells. BMC Cancer 2002; 2:27–35.
12. 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;21:1619–22.
13. Schneider Y, Duranton B, Gosse F, Schleiffer R, Seiler N, Raul F. Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis. Nutr Cancer 2001;39:102–7.
14. Ziegler CC, Rainwater L, Whelan J, McEntee MF. Dietary resveratrol does not affect intestinal tumorigenesis in Apc(*Min*/+) mice. J Nutr 2004;134:5–10.
15. Mutoh M, Takahashi M, Fukuda K, Matsushima-Hibiya Y, Mutoh H, Sugimura T, Wakabayashi K. Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure. Carcinogenesis 2000;21:959–63.
16. Maccarrone M, Lorenzon T, Guerrieri P, Agro AF. Resveratrol prevents apoptosis in K562 cells by inhibiting lipoxygenase and cyclooxygenase activity. Eur J Biochem 1999;265:27–34.
17. Martinez J, Moreno JJ. Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. Biochem Pharmacol 2000;59:865–70.
18. Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. Clin Chim Acta 1995;235:207–19.
19. Li ZG, Hong T, Shimada Y, Komoto I, Kawabe A, Ding Y, Kaganoi J, Hashimoto Y, Imamura M. Suppression of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. Carcinogenesis 2002;23:1531–6.
20. Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, Jang MS, Pezzuto JM, Dannenberg AJ. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J Biol Chem 1998;273:21875–82.
21. Zhang YJ, Jayaprakasam B, Seeram NP, Olson LK, DeWitt D, Nair MG. Insulin secretion and cyclooxygenase enzyme inhibition by Cabernet Sauvignon grape skin compounds. J Agr Food Chem 2004;52:228–33.
22. Johnson JL, Maddipati KR. Paradoxical effects of resveratrol on the two prostaglandin H synthases. Prostaglandins Other Lipid Mediat 1998;56:131–43.
23. Szewczuk LM, Forti L, Stivala LA, Penning TM. Resveratrol is a peroxidase-mediated inactivator of COX-1 but not COX-2: a mechanistic approach to the design of COX-1 selective agents. J Biol Chem 2004;279:22727–37.
24. Hansen-Petrik MB, McEntee MF, Jull B, Shi H, Zemel MP, Whelan J. Prostaglandin E(2) protects intestinal tumors from nonsteroidal anti-inflammatory drug-induced regression in Apc(*Min*/+) mice. Cancer Res 2002;62:403–8.
25. Uefuji K, Ichikura T, Mochizuki H. Expression of cyclooxygenase-2 in human gastric adenomas and adenocarcinomas. J Surg Oncol 2001;76:26–30.
26. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Upregulation of cyclooxygenase-2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107:1183–8.
27. Hull MA, Booth JK, Tisbury A, Scott N, Bonifer C, Markham AF, Coletta PL. Cyclooxygenase 2 is up-regulated and localized to macrophages in the intestine of Min mice. Br J Cancer 1999;79:1399–405.
28. Boolbol SK, Dannenberg AJ, Chadburn A, Martucci C, Guo XJ, Ramonetti JT, AbreuGoris M, Newmark HL, Lipkin ML, DeCosse JJ, Bertagnolli MM. Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. Cancer Res 1996;56:2556–60.
29. Ritland SR, Gendler SJ. Chemoprevention of intestinal adenomas in the Apc(Min) mouse by piroxicam: kinetics, strain effects and resistance to chemosuppression. Carcinogenesis 1999;20:51–8.
30. Jacoby RF, Marshall DJ, Newton MA, Novakovic K, Tutsch K, Cole CE, Lubet RA, Kelloff GJ, Verma A, Moser AR, Dove WF. Chemoprevention of spontaneous intestinal adenomas in the Apc (Min) mouse model by the nonsteroidal anti-inflammatory drug piroxicam. Cancer Res 1996;56:710–4.
31. Jacoby RF, Seibert K, Cole CE, Kelloff G, Lubet RA. The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. Cancer Res 2000;60:5040–4.
32. Kim S, Ko H, Park JE, Jung S, Lee SK, Chun YJ. Design, synthesis, and discovery of novel trans-stilbene analogues as potent and selective human cytochrome P4501B1 inhibitors. J Med Chem 2002; 45:160–4.
33. Chun YJ, Ryu SY, Jeong TC, Kim MY. Mechanism-based inhibition of human cytochrome P450 1A1 by rhapontigenin. Drug Metab Dispos 2001;29:389–93.
34. Lu JB, Ho CT, Ghai G, Chen KY. Resveratrol analog, 3,4,5,4′-tetrahydroxystilbene, differentially induces pro-apoptotic p53/Bax gene expression and inhibits the growth of transformed cells but not their normal counterparts. Carcinogenesis 2001;22:321–8.
35. Nam KA, Kim S, Heo YH, Lee SK. Resveratrol analog, 3,5,2′,4′-tetramethoxy-trans-stilbene, potentiates the inhibition of cell growth and induces apoptosis in human cancer cells. Arch Pharmacal Res 2001; 24:441–5.
36. She QB, Ma WY, Wang MF, Kaji A, Ho CT, Dong ZG. Inhibition of cell transformation by resveratrol and its derivatives: differential effects and mechanisms involved. Oncogene 2001;22:2143–50.
37. Potter GA, Patterson LH, Burke MD, Butler PC. Aromatic hydroxylation activated prodrugs. Int Pat Appl No WO 99/40056, 1999.
38. Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, Embleton J, Newell DR, Raymond R, Stables R, Stephens T, Wallace J. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (second edition). Br J Cancer 1998;77:1–10.
39. Luongo C, Moser AR, Gledhill S, Dove WF. Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res 1994;54:5947–52.
40. Strumia MM, Sample AB, Hart ED. An improved micro hematocrit method. Am J Clin Pathol 1954;24:1016–24.
41. Marsh KA, Stamp GWH, Kirkland SC. Isolation and characterization of multiple cell types from single human colonic carcinoma: tumourigenicity of the cell types in a xenograft system. J Pathol 1993; 170:441–50.
42. Plummer SM, Holloway KA, Manson MM, Munks RJL, Kaptein A, Farrow S, Howells L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signaling complex. Oncogene 1999;18:6013–20.
43. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
44. Takeda H, Sonoshita M, Oshima H, Sugihara K, Chulada PC, Langenbach R, Oshima M, Taketo MM. Cooperation of cyclooxygenase 1 and cyclooxygenase 2 in intestinal polyposis. Cancer Res 2003;63: 4872–7.
45. Seno H, Ishima M, Ishikawa T, Oshima H, Takaku K, Chiba T, Narumiya S, Taketo MM. Cyclooxygenase 2- and prostaglandin E_2 receptor EP_2-dependent angiogenesis in $Apc^{\Delta 716}$ mouse intestinal polyps. Cancer Res 2002;62:506–11.
46. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans J,F, Taketo MM. Suppression of intestinal polyposis in $Apc^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase-2 (COX-2). Cell 1996;87:803–9.
47. Keller JJ, Giardiello FM. Chemoprevention strategies using NSAIDs and COX-2 inhibitors. Cancer Biol Ther 2003;2:S140–9.
48. Gescher AJ, Steward WP. Relationship between mechanisms, bioavailibility, and preclinical chemopreventive efficacy of resveratrol: a conundrum. Cancer Epidemiol Biomarkers Prev 2003;12:953–7.
49. Zaveri NT, Waleh N, Chao WR, Bensari A. Inhibition of VEGF expression and tumor cell growth in breast cancer cells by novel synthetic analogs of the green tea catechin, epigallocatechin-3-gallate (EGCG). Cancer Epidemiol Biomarkers Prev 2003;12:1347S–8S.
50. Jong L, Chao WR, Amin K, Rice G. SRI 13668: a highly optimized AKT inhibitor based on indole-3-carbinol possesses potent oral anticancer activity, and induces G(1) cell cycle arrest and apoptosis *in vitro* and *in vivo*. Cancer Epidemiol Biomarkers Prev 2003;12:1348S.