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Epidemiological studies suggested that trans-resveratrol, a wine grape component, could prevent malignant tumor development. This compound also demonstrated cytostatic and cytotoxic effects on tumor cells in vitro. To obtain trans-resveratrol derivatives with a better cellular uptake and enhanced antiproliferative effects, we synthesized a triacetate derivative as well as an oligomer, ε-viniferin and its acetylated form, ε-viniferin penta-acetate. We also obtained vineatrol, a wine grape shoot extract that may act synergistically, inducing trans-resveratrol and ε-viniferin. We show here that resveratrol triacetate and vineatrol are as efficient as trans-resveratrol in inducing the accumulation of human colon cancer cells in early S phase of the cell cycle. This effect is associated with a nuclear redistribution of cyclin A and the formation of a cyclin A/cyclin-dependent kinase 2 complex whose kinase activity is increased. In contrast, ε-viniferin and its acetylated form do not demonstrate any significant activity on these cells when tested alone. Interestingly, resveratrol triacetate and vineatrol dramatically enhance 5-Fluoro-Uracil-mediated inhibition of colon cancer cell proliferation. Thus, acetylated derivatives of resveratrol have retained the cytostatic and cytotoxic activities of the parental molecule and thus deserve to be tested as chemosensitizers in animal models.

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Key words: colon cancer; cell cycle; polyphenols; resveratrol; vineatrol; chemosensitization

Epidemiological studies suggested that the polyphenol resveratrol (trans-3,4',5-trihydroxystilbene) was one of the main wine grape components that protect from vascular diseases, neurodegenerative processes and cancers.1 The ability of resveratrol to prevent the occurrence of carcinomas was related to the inhibition of tumor cell cycle,2,3 and induction of tumor cell death.4,5 In addition, there is compelling in vitro evidence that trans-resveratrol behaves as a chemosensitizer when combined with cytotoxic drugs,6 cytokines and other polyphenols such as quercetin, apigenin and genistein.6,7 Resveratrol is probably not the only bioactive compound present in the grape wine. Oligomers of resveratrol such as viniferins, were suggested to block the proliferation of several tumoral cell lines10-12 and a vineatrol preparation containing both trans-resveratrol and ε-viniferin exhibited a greater antiproliferative effect on malignant cell lines than each compound tested separately.13,14 Lipophilic derivatives were obtained through esterification of the hydroxyl functions with aliphatic molecules, which improved their intestinal absorption and cell penetration.15,16 Acetylation of the phenolic compounds could also favor their absorption, as demonstrated for catechins.17 Acetylated derivatives of polyphenols can be found in natural sources such as plants.18-21 The ability of acetylated polyphenols to promote tumor cell cycle arrest, death and to sensitize them to other anticancer agents remained to be explored. In the present study, we compare the effects of trans-resveratrol obtained from wine shoots (R), the acetylated form of trans-resveratrol (trans-resveratrol triacetate, R3A), the resveratrol oligomer ε-viniferin (εV) and its penta-acetate derivative (εV5A) and the vineatrol preparation (Vinea). We demonstrate that trans-resveratrol and its triacetate derivative have similar cell cycle effects on human colon cancer cell lines whereas the oligomer ε-viniferin does not exert any significant activity by itself. Acetylated polyphenols also demonstrate a synergistic effect with 5-Fluoro-Uracil.

Material and methods

Cell line

SW480, SW620 and HCT116 human colon carcinoma cell lines obtained from the American Tissue Culture Collection (ATCC, Rockville, MD) were maintained in RPMI 1640 medium for SW480 and SW620, HCT116 in Eagle’s minimum essential medium, both media were complemented with 10% fetal calf serum (Sigma-Aldrich, Saint Quentin Fallavier, France).

Drugs, chemical reagents and antibodies

Five compounds or preparations were used including trans-resveratrol obtained from vine shoots (R), trans-resveratrol triacetate (R3A), ε-viniferin (εV), ε-viniferin pentaacetate (εV5A) (Actichem, Montauban, France) and vineatrol, a mixture of polyphenols derived from vine shoots that contains 16% trans-resveratrol and 20% ε-viniferin.13 Briefly, vine-shoots are ground, extracted with acetone and concentrated, then diluted in an ethanol/water mixture, which is filtered, evaporated and submitted to a preparative HPLC. Resveratrol was purified by flash chromatography and identified by comparison with a standard from Sigma on a HPLC with a diodes detector. Viniferin was purified by flash semi-preparative chromatography, then HPLC. The identification was carried out by NMRH, Mass spectrometry and 2D. A stock solution of these polyphenols was prepared in ethanol. Stock solutions of vineatrol preparation were prepared with respect to their content in trans-resveratrol, corresponding to 23.8 mM resveratrol and 18.9 mM ε-viniferin.14 We used rabbit polyclonal antibodies (Abs) against cyclin A, cyclin B1 and cyclin E and mouse monoclonal Abs against Cdk1, Cdk2 (Santa-Cruz Biotechnology, Santa-Cruz, CA) and β-actin (Sigma-Aldrich).

Cell cycle analysis by flow cytometry

SW480 cells were seeded into 25 cm² flasks (6 × 10⁵ cells) in 4 mL of culture medium. After 24 hr of culture, the cells were treated in triplicate flasks with 30 μL of the polyphenols or with

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by a 15 min centrifugation at 20,000 × g. At indicated times, cells were labeled with 30 μg/mL BrdUrd (Sigma-Aldrich) for 1 hr at 37°C. Briefly, detached and adherent cells were pooled, washed in PBS and fixed in cold 70% ethanol for 2 hr, washed twice in PBS and incubated in a solution containing 0.2 mg pepsin in HCl 2 N at room temperature for 1 hr, rinsed with PBS and incubated with a mouse anti-BrdU monoclonal antibody (Dako, Denmark) for 1 hr at room temperature. The cells were washed again and incubated with a FITC-conjugated goat anti-mouse antibody (Dako, Denmark) for 40 min in the dark, rinsed with PBS and counterstained by a 50 μg/mL solution of propidium iodide containing 200 μg/mL RNase A. Stained cells were analyzed with a Galaxy flow cytometer (Partec, Münster, Germany) equipped with a 488 nm blue laser providing acquisition of 20,000 events. Red fluorescence from PI was detected through a 625 nm long pass filter (FL3, DNA content) and green fluorescence from FITC was detected through a 520/10 nm band pass filter (FL1, BrdUrd incorporation). The acquired data were analyzed with FlowMax software (Partec) and displayed as dual-parameter PI versus log-FITC histograms. Three major compartments were first identified (BrdUrd-positive S-phase cells, BrdUrd-negative G1 and G2/M phases). Then, BrdUrd positive cells were divided into 3 subpopulations according to their DNA content. Electronic gating on BrdUrd negative populations (G1 and G2/M cells) allowed obtaining bivariate side-scatter/DNA content diagrams of these cells. Analyses by these parameters permitted to distinguish the G1a, G1b, G2 and M phases, which present different light scatter properties.

Immunofluorescence analyses of cyclins expression

SW480 cells were seeded into 25 cm² flasks (6 × 10⁵ cells) in 4 mL of culture medium. After 24 hr, cells were treated in triplicate with 30 μM polyphenols or vineatrol. At indicated times, cells were collected, washed in PBS and fixed in 70% ethanol for 2 hr at 4°C, then incubated either with rabbit anti-cyclin A or anti-cyclin B1 in 0.1% saponin in PBS containing 1% BSA for 1 hr at room temperature. Negative controls were treated with irrelevant rabbit primary antibody. Cells were washed in PBS and rabbit primary antibodies were revealed with a 488-Alexa goat anti-rabbit antibody (Molecular Probes, Eugene, OR) diluted in PBS. Washed cells were counterstained with PI. Flow cytometric analysis was performed through the FL1 channel for 488-alexa green fluorescence and the FL3 channel for PI fluorescence. Expression levels of cyclins were plotted as diagrams of events number related to 488-alexa fluorescence in treated cells against control. Data were also presented as cyclin expression related to DNA content.

Immunocytochemistry studies

Tumor cells were seeded into tissue culture chambers at 20,000 per well (Chamber Slide, Life Technologies) for 24 hr, then treated and subsequently fixed in 2% paraformaldehyde (Sigma-Aldrich) for 10 min at 4°C, washed twice with PBS for 10 min, then preincubated with 1% bovine serum albumin for 15 min at room temperature and incubated with the primary Ab (PBS, 0.1% saponin, 0.5% bovine serum albumin) for 2 hr, washed and incubated for 30 min with 488-alexa goat anti-rabbit or 568-alexa goat anti-rabbit Ab (Molecular Probes, Eugene, OR). Nuclei were counterstained with PI. Flow cytometric analysis was performed with a Galaxy flow cytometer (Partec, Münster, Germany) equipped with a 488 nm blue laser providing acquisition of 20,000 events. Red fluorescence from PI was detected through a 625 nm long pass filter (FL3, DNA content) and green fluorescence from FITC was detected through a 520/10 nm band pass filter (FL1, BrdUrd incorporation). The acquired data were analyzed with FlowMax software (Partec) and displayed as dual-parameter PI versus log-FITC histograms. Three major compartments were first identified (BrdUrd-positive S-phase cells, BrdUrd-negative G1 and G2/M phases). Then, BrdUrd positive cells were divided into 3 subpopulations according to their DNA content. Electronic gating on BrdUrd negative populations (G1 and G2/M cells) allowed obtaining bivariate side-scatter/DNA content diagrams of these cells. Analyses by these parameters permitted to distinguish the G1a, G1b, G2 and M phases, which present different light scatter properties.

Immunoprecipitation

SW480 cells were seeded into 80 cm² flasks (2 × 10⁶ cells) for 24 hr, then treated with polyphenols or vineatrol. After 48 hr, detached and adherent cells were recovered, washed in PBS and incubated in a lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM Na₂P₂O₇, 100 mM NaF, 2 mM Na₃VO₄, 1% Triton-X 100) complemented with antiproteases for 15 min on ice. After centrifugation at 20,000 g at 4°C for 20 min, 500 μg of proteins from the supernatant were incubated overnight at 4°C with 2 μg of a mouse anti-Cdc2 or anti-Cdk2 in 1 mL HNT buffer (Heps 30 mM, NaCl 30 mM, 0.1% Triton-X 100). Immune complexes were precipitated using protein G-Sepharose (Pharmacia, Orsay, France) during 2 hr at 4°C and washed 3 times in HNT buffer. The precipitates were resuspended in a loading buffer and boiled for 5 min. Samples were resolved on SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad) for western blot analysis.

Cyclin/Cdk complexes activities measurements

Kinase activities of cyclins-Cdk complexes were measured as described with some adaptations. Briefly, 300 μg of proteins extracted as described above were immune-precipitated with rabbit anti-cyclin A, B1 or E. The immune complexes were washed 3 times in a kinase buffer (50 mM Hepes pH 7.5, 10 mM MgCl₂, 250 μM EGTA, 10 mM β-glycerophosphate, 100 μM Na₃VO₄, 1 mM DTT) and resuspended in 30 μL of kinase buffer. Kinase reaction was performed by adding 20 μL of a mixture containing 0.3 μM ATP (Sigma-Aldrich, France) and 2 μg histone H1 (Calbiochem-VWR, Strasbourg, France) as substrate. After 30 min at 37°C, an equal volume of kinase-GLO reagent (Promega, France) was added. Controls were kinase reactions in the absence of the substrate. After 10 min at room temperature, luminescence was recorded using a FLX-800 microplate reader (Bio-Tek Instruments). Light emission is proportional to unconsumed ATP and inversely proportional to kinase activity. Background luminescence was corrected.
cence measured without substrate was subtracted from total luminescence and kinase relative activities were calculated. Purities of immune complexes were determined by Ponceau Red staining and western blot analyses.

Polyphenols chemosensitization assays with 5-FU
SW480 cells (1 × 10^5) were seeded into 24-well plates in 1 mL of culture medium. After 48 hr, the cells were left untreated or pretreated for 24 hr in triplicate wells with polyphenols or vineatrol, then exposed for 24 hr to 5-FU. Adherent cells and detached cells were then collected and pooled and cell growth was determined by counting viable cells by trypan blue dye exclusion with a hemocytometer.

**Statistical analysis**
All data are the mean ± SD of at least 3 independent experiments whose significance was established with the Student’s test.

**Results**
**Tumoral colon cells accumulation in early S phase by resveratrol analogs and vineatrol**
We first compared the cell cycle effects of the studied polyphenols at 30 μM, a concentration previously shown to arrest cell cycle progression before inducing cell death. SW480 cells were exposed to 30 μM of trans-resveratrol obtained from wine shots (R), trans-resveratrol triacetate (R3A), e-viniferin (eV) and e-viniferin pentaacetate (eV5A) for 72 hr (Fig. 1). Cells were also
exposed to vineatrol containing either 10 μM (Vinea 10 including 8 μM ε-viniferin) or 30 μM (Vinea30 including 24 μM ε-viniferin) trans-resveratrol for 24 to 72 hr before addition of BrdUrd in the last hour of treatment and cell cycle analysis by flow cytometry. Cells with high fluorescence were S phase cells that incorporated BrdUrd. Those with background fluorescence (Fig. 2a) represented G1 or G2/M phase cells, depending on DNA content (2C or 4C, respectively).

After 24 hr of treatment with R, R3A and Vinea30, the percentage of cells in S phase was increased (Fig. 2b). This effect was enhanced with time and was associated with a decrease in the percentage of cells in G1 phase and a virtually complete disappearance of cells in G2/M (Figs. 2a and 2b). Vinea10 induced a slighter increase in S phase, suggesting that the cell cycle effects of these drugs were dose-dependent (Figs. 2a and 2b). R3A and Vinea demonstrated similar effects on HCT116 and SW620 colon cancer cell lines (supplementary data). Conversely, eV and eV5A tested alone demonstrated limited effects on the cell cycle in all the studied colon carcinoma cell lines (Fig. 2b and supplementary data).

To further analyze the dysregulation of S phase in polyphenol-induced cell cycle arrest, we distinguished 3 populations among the BrdUrd incorporating cells. Those with a DNA content between 2.0 and 2.3C are entering S phase (early-S: S_E), those with a DNA content between 2.3 and 3.8C were in the middle of S phase (mid-S: S_M) and those with a DNA content between 3.8C and 4.0C were exiting S phase (late-S: S_L) (Fig. 2a). A 24 hr exposure to R, R3A and Vinea30 induced a 15% increase in the percentage of cells in S_E. This increase reached 24% after 72 hr of drug exposure (Fig. 2c). Again, comparison of Vinea10 and Vinea30 indicated a dose-dependent effect (Fig. 2c). These observations suggested that R, R3A and vineatrol induced accumulation of tumor cells into early S phase.

We therefore gated on BrdUrd negative cells and analyzed forward and light scatter parameters (Fig. 3a). The 90º light scattering exhibited by cells in G2 was almost twice that of cells in M phase, which was confirmed by cell sorting and analysis of the mitotic index (not shown). S phase accumulation induced by R and R3A led to a strong decrease in the percentage of cells into M phase (Fig. 3b). Cells into G1 phase were also subdivided into 2 compartments, i.e., G1A representing post-mitotic cells and G1B cells ready to initiate DNA synthesis. R, R3A and Vinea30 induced a time-dependent increase in G1A and a decrease in G1B (Fig. 3b). Again, this analysis did not detect any effect of eV and eV5A on the cell cycle (Fig. 3b).

**Resveratrol analogs and vineatrol favor cyclin A accumulation**

These observations led us to explore whether cyclins and their kinases, the cyclin-dependent kinases (Cdks), could be affected upon exposure to the studied compounds. Immunoblotting and flow cytometry analyses demonstrated that R (30 μM), R3A (30 μM) and Vinea10 dramatically increased cyclin A expression (Figs. 4a and 4b), especially in early S phase (supplementary data). A concomitant cyclin B expression was observed in a time-dependent manner (Figs. 4a and 4b). Immunoblot analyses detected also a time-dependent increase in cyclin E as well as in Cdk1 and Cdk2 protein levels (Fig. 4a). Similar results were obtained in HCT116 and SW620 cells (supplementary data). Again, eV and eV5A did not demonstrate any effect in these experiments.

**Cyclin A relocalization-induced by resveratrol analogs and vineatrol**

The increase in cyclin A expression upon exposure to polyphenols for 48 hr was confirmed by immunofluorescence studies (Fig. 5a). Nuclear staining of colorectal cells demonstrated that resveratrol-analogs and vineatrol preparation induced a nuclear redistribution of cyclin A that might precede apoptosis as it was found in both normal and apoptotic cells (Fig. 5a). A slight increase in cyclin B that also re-localized in the nucleus upon exposure to R,
R3A and Vinea was confirmed (Fig. 5b). Upon exposure to R, R3A and Vinea, colon carcinoma cells underwent apoptosis in a dose- and time-dependent manner (Fig. 5c). Again, cyclin A redistribution and apoptosis induction were not observed upon exposure to eV and eV5A (Figs. 5a and 5c).

**Resveratrol analogs and vineatrol disturb cyclin/Cdk complex activities**

Co-immunoprecipitation experiments using anti-Cdks antibodies demonstrated that R, R3A and vineatrol (30 μM; 48 hr) promoted interaction of Cdk2 with cyclins A and E (Fig. 6a) and Cdk1 binding to cyclin B (Fig. 6b). These results were confirmed by reverse co-immunoprecipitations (Fig. 6c). These treatments also increased cyclin A/Cdks kinase activities (Fig. 6c), as measured by ATP-consumption associated with phosphorylation of H1 histone, a cyclins/Cdks substrate. Conversely, we failed to detect any significant increase in cyclin E/Cdk2 and cyclin B/Cdk1 kinase activities. Surprisingly enough, eV, eV5A and low-concentration of vineatrol (Vinea10) induced cyclin B/Cdk1 activity.

**Synergistic effect of vineatrol and resveratrol-analogs with 5-FU on colon cancer cells**

As 5-FU is mainly active in S phase cells, we looked for a synergistic effect of the combination of resveratrol analogs and vineatrol with this classically used cytotoxic agent in colon cancer treatment. Pretreatment with R, R3A (10 μM) or Vinea10 for 24 hr dramatically enhanced inhibition of SW480 cell proliferation upon 5-FU treatment (Fig. 7). Polyphenolic pretreatment cooperates with the anticancer drug, 5-FU, to kill cancer cells in a dose-dependent manner (Fig. 7). Co-treatments with both 5-FU and polyphenolic compounds were significantly less effective than treatment with resveratrol followed by cytotoxic drugs and only slightly more effective than treatment with either low cytotoxic drug concentration or resveratrol alone (Fig. 7). These findings indicate that pretreatment with low concentration (10 μM) of
resveratrol, its acetylated derivatives and vineatrol sensitize colon cancer cells to 5-FU antiproliferative and cytotoxic effects.

**Discussion**

The present study demonstrates that acetylated analogs of resveratrol as well as the mixture of polyphenolic compounds

**FIGURE 5** – Microscopic analyses of Cyclins A and B1 subcellular localization. SW480 cells were treated as in figure 2 for 48 hr, then stained for cyclin A (a) or cyclin B1 (b) detection by immunofluorescence and counterstained with Hoechst 33342 (Blue). White arrows show apoptotic nuclei. Apoptotic cells number percentages were estimated by counting 300 nuclei in triplicate slides: normal, fragmented (F), condensed (C). Means ± SD (n = 4) of 3 independent experiments. Percentages of apoptotic cells after treatment with R, R3A, Vinca 10 and 30 are statistically different from that of untreated cells (p < 0.05; Student’s test).

**FIGURE 6** – Resveratrol analogs and vineatrol disturb cyclin/Cdk complex formation and subsequent activities. SW480 cells were treated as in Figure 2 during 48 hr and cell proteins were submitted to immunoprecipitation with anti-Cdk2 (a) or anti-Cdk1 (b) antibody. Western blot analyses reveal complexation of these kinases with their regulatory cyclins subunits. Protein loading: anti-Cdks antibodies. (c) Total proteins obtained from treated cells (see above) were immunoprecipitated for cyclin A, B1 and E complexes. Relative kinase activities of these complexes were recorded by measuring their ATP consumption to phosphorylate histone H1. Purity of samples was evaluated by western blot analyses against Cdks proteins. Mean ± SD of triplicate measures of one representative among 3 independent experiments. *Means significantly differ from the control with p < 0.05 by Mann-Whitney test.
known as vineatrol affect cell cycle progression of human colon cancer cell lines and favor accumulation of these cells in early S phase, which sensitizes them to 5-FU cytotoxic effects. Thus, these resveratrol derivatives have similar effects on tumor cells as the parental molecule. We also demonstrate that eV oligomers, even if their acetylated form (eV5), have limited if any activity on tumor cell progression in the cell cycle and apoptotic death.

Resveratrol was proposed to function as a cancer chemopreventive agent through inhibition of promutagen bioactivation and stimulation of carcinogen detoxification. Resveratrol and some derivatives also possess cytostatic and cytotoxic effects in tumor cells in vitro. We recently developed acetylated forms of resveratrol that still inhibit colon cancer cell proliferation and this acetylation can improve its cellular uptake. The combination of resveratrol with other polyphenols could favor inhibition of cell proliferation and vineatrol preparation that combines trans-resveratrol and e-viniferin could exhibit a greater antiproliferative effect than resveratrol alone in various tumor cells tested in vitro.

Here, we show that purified trans-resveratrol, acetylated trans-resveratrol and vineatrol all arrest human colon cancer cells in G1B and early S phase with a similar efficacy. These compounds induce a significant increase of cyclin A expression that accumulates in the nucleus to form a complex with Cdk2. Cyclin A and Cdk2 might be responsible for activating components of the DNA replication initiation complex on entry into S phase. Cyclin B1 was also relocated in the nucleus and formed a CyclinB/Cdk1 complex and Cyclin E accumulated and interacted with Cdk2. Altogether, these events may favor DNA replication. This increase in cell cycle key protein regulators and the subsequent accumulation of tumor cells into S phase preceded apoptotic cell death. Cyclin A accumulation in tumor cells was proposed to predict a positive response to classical chemotherapy. Accordingly, the death induction was amplified when resveratrol analogs and vineatrol were combined with the anticancer agent 5-FU. This later drug, which affects the biosynthesis of nucleic acids into S phase, is one of the most widely used agents in colon cancer chemotherapy. Pretreatment with trans-resveratrol, R3A or vineatrol could reduce the dose of 5-FU, thus limiting its toxicity, or improving its efficacy at therapeutic doses.

At least 2 events could account for the chemosensitizing effect of resveratrol as a pretreatment. First, resveratrol induces the accumulation of colon cancer cells in S phase, which may significantly account for the increased efficacy of 5-FU. Second, resveratrol could affect the lysosomal function and reduce cell environment acidity in a manner similar to that described for the proton pump inhibitor omeprazole. Resveratrol has been shown to inhibit the H+-ATP synthase through targeting the F(1) catalytic domain. A pre-treatment with resveratrol and its analogs could therefore reduce intracellular acidity and facilitates the cytotoxic drug action. This innovative combination may be more efficient than using a single drug at higher concentration.

Several studies have shown that polyphenols have no cytotoxic effect on normal cells and at the concentrations inducing growth inhibition and cell cycle arrest in colon carcinoma cells, polyphenol compounds did not impair the viability of human normal peripheral blood mononuclear cells (PBMC) and much higher concentrations were required to induce a cytotoxic effect in these cells.

![Figure 7](image-url) - Synergistic effect of some resveratrol analogs with 5-Fluoro-uracile. SW480 cells (1 × 10^5) were seeded into 24-well plates in 1 mL of culture medium. After 48 hr, the cells were left untreated or pretreated for 24 hr with R (a) and R3A (b) (10 μM) or with Vinea 10 (10 μM resveratrol) (c). Pretreated cells were then exposed for 24 hr to 5-FU at indicated concentrations. Cells were also cotreated with polyphenols and 5-FU. The cell growth extent was determined by counting viable cells by trypan blue dye exclusion with a hemocytometer. Percentage of viable cells in treated against control ± SD (n = 5, 3 independent experiments).
cells. Moreover, several reports showed that resveratrol could prevent or delay the onset of cancer. These effects are observed despite extremely low bioavailability and rapid clearance from the circulation, which explains why many laboratories observed with resveratrol in vitro. In our hands, oligomers of resveratrol such as e-viniferin, even in an acetylated form, did not reproduce the reveratrol effects in vitro and their association to resveratrol in vineatrol does not potentiate its efficacy. Additional studies are now in progress to determine the impact of the synergy between acetylated forms/5-FU on animal models of colon carcinogenesis.

**References**


