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RESVERATROL ANALOG (*Z*)-3,5,4′-TRIMETHOXYSTILBENE IS A POTENT ANTI-MITOTIC DRUG INHIBITING TUBULIN POLYMERIZATION

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Resveratrol (3,5,4′-trihydroxystilbene) a natural polyphenol present in medicinal plants, grapes and wines, has potent chemopreventive properties on intestinal carcinogenesis. A methylated derivative (*Z*-3,5,4′-trimethoxystilbene: R3) was synthesized. R3 at 0.3 μM exerted a 80% growth inhibition of human colon cancer Caco-2 cells and arrested growth completely at 0.4 μM (R3 was 100-fold more active than resveratrol). The *cis* conformation of R3 was also 100-fold more potent than the *trans* isomer. R3 (0.3 μM) caused cell cycle arrest at the G2/M phase transition. The drug inhibited tubulin polymerization in a dose-dependent manner (IC_{50}= 4 μM), and it reduced also by 2-fold ornithine decarboxylase and s-adenosylmethionine decarboxylase activities. This caused the depletion of the polyamines, putrescine and spermidine, which are growth factors for cancer cells. R3 inhibited partially colchicine binding to its binding site on tubulin, indicating that R3 either partially overlaps with colchicine binding or that R3 binds to a specific site of tubulin that is not identical with the colchicine binding site modifying colchicine binding by allosteric influences. The resveratrol derivative (*Z*)-3,5,4′-trimethoxystilbene (R3) is an interesting anti-mitotic drug that exerts cytotoxic effects by depleting the intracellular pool of polyamines and by altering microtubule polymerization. Such a drug may be useful for the treatment of neoplastic diseases.

Key words: *Caco-2 cells; colon cancer; cell cycle; polyamine; tubulin; colchicine*

Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenol present in a variety of medicinal plants and in grapes.[1] Its function is to protect plants against pathogenic attack and environmental stress.[2,3] Because of its production by grape skin, significant amounts are found in red wines.[4] First evidence of the beneficial effects of resveratrol for human health was shown by its ability to protect against cardiovascular diseases. Resveratrol exhibits strong antioxydant activity[5,6] and inhibits platelet aggregation.[7,8] More recently, numerous cancer chemopreventive properties of resveratrol have been demonstrated .[9–12]

Ptero-stilbene, a derivative of resveratrol that is methylated at positions 3 and 5, exhibits greater antifungal activity than resveratrol.[13,14] However, this molecule is only present in very low concentrations in grapes and seems to be a minor effector of the plant defensive response. It has been shown that methylated derivatives of flavonoïds exhibit higher antiproliferative potency on cancer cells than their hydroxylated counterparts.[15] This ability may be related to the increased lipophilic properties of the methoxyflavonoids and their increased uptake through the cell membrane. On this basis we have developed a methylated derivative of resveratrol (R3: *Z*-3,5,4′-trimethoxystilbene; Fig. 1) with the aim to evaluate its antiproliferative effects on the human colon cancer cell line Caco-2.

Previously resveratrol was shown to inhibit the proliferation of Caco-2 cells through the accumulation of cells in the S phase of the cell cycle and by inhibiting polyamine biosynthesis.[16] The effects of resveratrol were not cytotoxic but mainly cytostatic and reversible. In the present study, we show that R3 exerts a 100-fold higher inhibitory effect on colon cancer cell growth. The growth supressive effects induced by R3 were totally different from those observed with resveratrol and were related to a selective blockade of cells at the G2/M phase of the cell cycle, and to the disruption of the microtubule network.

MATERIAL AND METHODS

Synthesis of (Z)-3,5,4.′′-trimethoxystilbene (R3)

The compound was synthesized by a Wittig-Horner reaction from 4-methoxybenzyl-diethyl phosphonate and 3,5-dimethoxybenzaldehyde[17,18] (Fig. 2). All reagents were commercially available and used without further purification.

Melting points were measured without correction in capillary tubes on a Büchi apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 MHz spectrometer. Mass spectra were established on a Fisons spectrometer at 70 eV chamber voltage, using a direct inlet tube. Silica gel 60 (40–63 mesh, Merck Eurolab SA, Strasbourg, France) and thin layer chromatography with aluminium sheets 20 × 20 cm 60 F 254 (Merck Eurolab SA, Strasbourg, France) were used. Dimethylformamide was distilled over 0.4 nm molecular sieves under reduced pressure prior to its use; 4-Methoxybenzyl alcohol (1) was purchased from Acros (Noisy le Grand, France), 3,5-dimethoxybenzaldehyde from Avocado (La Tour du Pin, France) and triethylphosphite from Sigma-Aldrich (St. Quentin Fallavier, France).

4-methoxybenzyl bromide (2). Seven milliliters of hydrobromic acid 48% (7 ml) was added to 4-methoxybenzyl alcohol (7.5 g = 54 mmoles) and the mixture was vigorously stirred at room temperature for 30 min. Diethyl ether (100 ml) was then added and the acid layer was separated. After a second extraction, the organic layers were combined, washed with a saturated solution of sodium hydrogenocarbonate, then with brine and finally dried over magnesium sulfate. The compound formed (2) was used without further purification in the next step (colorless oil, weight 10.54 g and yield 97%).

(E)-3,5,4′-trimethoxystilbene (3). A mixture of triethylphosphite (14.4 ml = 85 mmoles) and 4-methoxybenzyl bromide (10.54 g = 52.4 mmoles) was heated at 120°C for 6 hr. During the reaction, ethyl bromide evolved. After cooling at 0°C, dimethylformamide

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(75 ml) and sodium methylate (2.97 g = 55.2 mmoles) were added and stirred during 1 hr. After addition of 3,5-dimethoxybenzaldehyde (5.8 g = 35 mmoles), the solution was stirred for 1 hr at room temperature and another hr at 100°C. After cooling, the reaction mixture was stirred overnight before quenching with water and extraction with diethylether. The combined organic layers were washed with brine until litmus and dried over magnesium sulfate. The crude product was purified on silica gel using hexane and ethyl acetate (4:1) as eluent (colorless needles, weight 7.85 g and yield 83%). $C_{17}H_{18}O_3$ mp = 56–57°C, ^{1}H, $CDCl_3$, 200 MHz : 7.48 (2H, d, J = 9 Hz, $H_{2',6'}$); 7.00 (2H, d, J = 16.5 Hz, vinylics); 6.93 (2H, d, J = 9 Hz, $H_{3',5'}$) ; 6.68 (2H, d, J = 2,5 Hz, $H_{2,6}$); 6.40 (^{1}H, t, J= 2,5 Hz, H_4); 3.85 (9H, s, 3 xOMe), ^{13}C, $CDCl_3$, 200 MHz: 161 (C_3); 159.5 (C_5); 159.3 ($C_{4'}$); 139.7 (C_1); 130 ($C_{1'}$); 128.7 (C); 127.8 ($C_{2',6'}$); 126.6 (C); 114.2 ($C_{3'}$); 113.8 ($C_{5'}$); 104.4 ($C_{2,6}$); 99.7 (C_4); 55.4 (OCH_3x3), Mass. m/z = 270 (M^+, 100%) ; 258.0 ; 239.0 ; 228.1 ; 197.1 ; 150.1 ; 137.0 ; 121.1 ; 109.1.

(Z)-3 ,5, 4′-trimethoxystilbene (R3) (4). Conversion of the *E* form into the *Z* form was achieved by U.V. irradiation (294 nm for 60 min).

Cell culture

Human colon adenocarcinoma CaCo-2 cells, obtained from the European Collection of Animal Cell Culture (Salisbury, UK), were cultured in 75 cm^2 Falcon flasks in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and subcultured to preconfluency after trypsinization (0.5% trypsin/2.6 mM EDTA). Cells were seeded either at 6×10^5 cells on culture dishes (100 mm in diameter) or at 4,500 cells per well on 96-well plates. For immunofluorescence studies cells were seeded at 3.5×10^4 cells per 14 mm glass coverslip. The culture medium was composed of DMEM supplemented with 3% horse serum, transferrin (5 μg/ml), selenium (5 ng/ml) and insulin (10 μg/ml) (TSI-defined medium; Gibco BRL, Life Technologies SARL, Cergy Pontoise, France). When needed, R3 diluted in dimethylsulfoxide (0.1% final) was added to the culture medium 24 hr after seeding. In all experiments, the culture medium was replaced every 48 hr and R3 was freshly added.

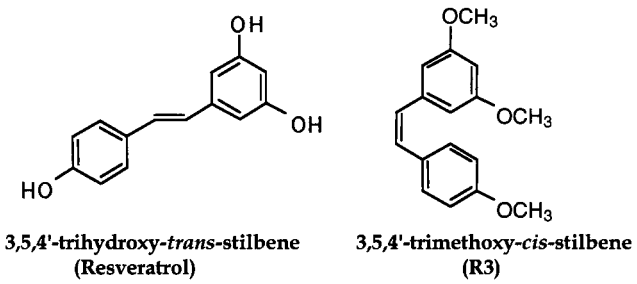

FIGURE 1 – Chemical structure of resveratrol and (*Z*-)3,5,4′-trimethoxystilbene (R3).

Cell growth

Cells were seeded in 96-well microplates and incubated for different times. Cell growth was stopped by addition of 50 μl trichloroacetic acid (50% v/v) and the protein content of each well was determined by staining with sulforhodamine B.[19] Absorbance was determined at 490 nm. The relationship between cell number (protein content/well) and absorbance is linear from 0 to 200,000 cells per well.

Determination of apoptosis and cytotoxicity

After trypsinisation, cells were collected by centrifugation and stored at −80 °C. Apoptotic DNA was separated from genomic DNA, using the Suicide Track™ DNA ladder kit (Oncogene Research Products, Cambridge, MA). The DNA fragments were separated by electrophoresis and stained with ethidium bromide.

Phosphatidylserine membrane externalization was measured using annexin V conjugated FITC binding (Annexin V-FITC kit, Medsystems Diagnostics Gmbh, Vienna, Austria). In brief, cells were washed in cold PBS without calcium. For each sample, 5×10^5 cells were resuspended in 100 μl of the reaction buffer [10 μl binding buffer, 10 μl of propidium iodide (PI), 1 μl of annexin V-FITC and 79 μl of deionized water]. After 15 min of incubation in the dark at room temperature, each sample was diluted with binding buffer to obtain a final volume appropriate for flow cytometry.

For the determination of cytotoxicity, cells (5,000 per well) were seeded in 96-well microplates and incubated in 3% horse serum-supplemented DMEM culture medium. Forty-eight hours after seeding, cells were incubated with various concentration of R3 or 0.1% DMSO for 6, 16 and 24 hr. Cytotoxicity was assessed by determining lactate dehydrogenase (LDH) release into the culture medium using the CytoTox 96® nonradioactive cytotoxicity assay kit (Promega, Charbonnières les Bains, France). The relationship between lysed cells and absorbance is linear from 0 to 100,000 cells per well; the number of lysed cells was proportional to the LDH activity measured in the culture medium.

Cell cycle analysis

Cell cycle phase distribution was analyzed by labeling cells with propidium iodide and assays were carried out as described previously.[20] Briefly, cells were harvested by trypsinization (0.5% trypsin/2.6 mM EDTA), washed twice with ice-cold PBS and fixed in methanol/PBS (9/1 v/v) at −20°C for at least 30 min. The fixed cells were then stained with 50 μg/ml of PI in the presence of 25 μg/ml of RNAse A. Cell cycle phase distribution was analyzed in 3 different experiments using FACS flow cytometry (FACS scan® flow cytometer, Becton Dickinson Immunocytometry Systems, San Jose, CA). Data from 10,000 events/sample were collected and

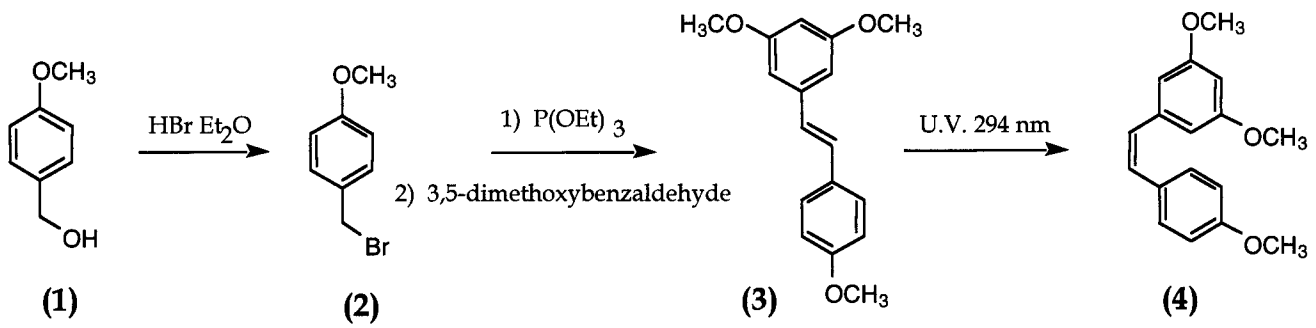

FIGURE 2 – Synthesis of (*Z*-)3,5,4′-trimethoxystilbene (R3).

analyzed using Cell Fit® cell analysis program (Becton Dickinson Immunocytometry Systems).

Immunofluorescence staining

Cells, cultured for 5 days, were incubated for 2 hr in DMEM containing either R3 (1 μM, 5 μM, 10 μM and 20 μM), Nocodazole (20 μM) or Taxol (10 μM). Cells were treated with 0.5% Triton X100 in MTBS (0.1 mM Pipes, pH 6.9; 1 mM EGTA, 4% polyethylene glycol, 0.02% and NaN_3) for 5 min before fixation, rinsed in MTBS and fixed in 100% methanol at −20°C for 5 min. For permeabilization, the fixed cells were treated for 15 min with 1% Triton X-100 in PBS. After washings in MTBS followed by PBS, cells were incubated for 40 min at room temperature in 5% goat serum dissolved in PBS and then incubated overnight at 4°C with mouse monoclonal anti-β-tubulin (Amersham Pharmacia Biotech, Saclay, France). After 3 washes with PBS, cells were incubated for 45 min with Alexa fluorTM488-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR). Finally, cells were rinsed in PBS and mounted in 1% (W/V) paraphenylenediamine dissolved in PBS/glycerol. All samples were examined with an Olympus AX60 fluorescence microscope.

Tubulin polymerization assay

Reaction mixtures contained 2.5 mg/ml of pure tubulin (Tebu International, Le Perray en Yvelines, France) that was diluted in G-PEM buffer (80 mM PIPES pH 6.9; 1 mM EGTA; 1 mM GTP and 10% glycerol) in the presence or absence of R3. Samples were placed in quartz cuvettes and incubated at 37°C. Tubulin polymerization was followed by measuring absorbance at 340 nm.

Colchicine and vinblastine binding assays

Cells were homogenized in 10 mM sodium phosphate, 10 mM $MgCl_2$, pH 6.8 (PM buffer). The homogenate was kept for 30 min on ice and then centrifuged at 100,000*g* for 60 min at 4°C to obtain the high-speed supernatant (cytosol). Colchicine binding assays were carried out by adding 100 μl of Ring C-[methoxy-$_3$H] colchicine (5 μCi/ml) (76.5 mCi/mmol, NEN, Boston, MA) to 200 μl of undiluted cytosol (containing at least 2 mg of protein) and 700 μl of PM buffer, supplemented or not with R3. Vinblastine binding assays were carried out by adding 100 μl of [G-^{3}H] vinblastine sulphate (0.5 μCi/ml) (9.6 mCi/mmol, NEN, Boston, MA).

Purified tubulin (5μM) was incubated with R3 and colchicine (1μM), spiked with Ring C-[methoxy-^{3}H] colchicine (5 μCi/ml) (76.5 mCi/mmol, NEN, Boston, MA) under similar conditions as described above in MES buffer pH 6.8 (100 mM Mes, 1 mM EDTA, 1 mM EGTA and 1 mM $MgCl_2$).

Samples were heated for 60 min at 37°C in a water bath before the reaction was terminated by transferring the tubes to an ice bath. After 5 min, each sample was collected onto stacks of 4 Whatman DE81 filter papers. Filters were washed, placed in a counting vial and radioactivity was measured by liquid scintillation spectrometry.

When tubulin was not present negligible levels of [^{3}H] colchicine were detected, indicating that the free (nontubulin-bound colchicine) compound retained on the filters.

ODC and AdoMetDC activities

Cells were homogenized in 100 mM Tris-HCl buffer, pH 7.4 (1 mM EDTA, 1 mM dithiothreitol, 0.5 μM leupeptin and 0.5 mM phenylmethylsulfonyl fluoride). After centrifugation at 33,000*g* for 25 min at 4°C, the supernatants were collected and ODC and AdoMetDC assays were performed. ODC activity was evaluated by measuring $^{14}CO_2$ formation from [1-^{14}C]L-ornithine (55 mCi/mmol, Amersham Pharmacia Biotech, Orsay, France)[21] and AdoMetDC activity was evaluated by measuring $^{14}CO_2$ formed from [1-^{14}C]S-adenosylmethionine (60 mCi/mmol, Amersham Pharmacia Biotech).[22]

Polyamine determinations

Cells were homogenized in perchloric acid (200 mM), and the homogenates were centrifuged at 1,500*g* for 10 min after standing for 16 hr at +2°C. The clear supernatants were diluted with perchloric acid (200 mM) and 200 μl aliquots were applied on a reversed-phase column for separation.

The polyamines (putrescine, spermidine and spermine) were determined by separation of the ion pairs formed with n-octanesulfonic acid, reaction of the column effluent with o-phthalaldehyde/2-mercaptoethanol reagent and monitoring of fluorescence intensity.[23]

Statistical analysis

All experiments were performed at least 3 times. Data are reported as mean ± SE. Statistical differences between control and treated cells were evaluated using the Student's *t*-test. Differences were considered significant at $p < 0.05$.

RESULTS

R3 and cell growth

In order to determine the optimal dose for the inhibition of Caco-2 cell growth, cells were treated with concentrations of R3 ranging from 0.1 to 1 μM. The medium was changed every 48 h and fresh R3 was added.

The inhibitory effects of R3 on Caco-2 cell growth were already observed at a concentration of 0.2 μM, and 80% growth inhibition was obtained at 0.3 μM (Fig. 3). At higher concentrations (ranging from 0.4 to 1 μM) R3 caused complete growth arrest. As illustrated in Table I, R3 exhibited similar growth inhibitory effects on Caco-2 cells and 2 other human colon cancer cell lines (SW480 and SW620) and it exhibited even higher activity against human head and neck cancer cells, and lymphoma cells. Maximum effects were observed only when R3 molecules were in the *cis* (or *Z*) conformation. This observation suggests that the inhibitory effects of R3 are related to its *Z* conformation conferring to that isomer a stronger inhibitory potential. For this reason, all further experiments were carried out with the *Z* isomer.

Effects of R3 on cell death

To determine whether R3 is cytotoxic, LDH release was measured. LDH is a stable cytosolic enzyme that is released upon cell lysis caused by mitochondrial alteration and plasma membrane disruption. After exposure of cells to R3 (0.3 μM) for 48 hr, no

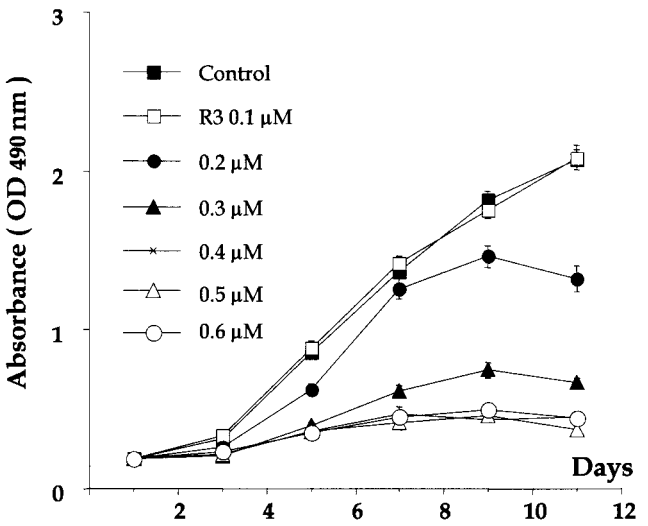

FIGURE 3 – CaCo-2 cell growth curves. Cells were seeded at 4,500 cells in 96-well plates in DMEM medium supplemented with 3% horse serum, transferrin (5 μg/ml), selenium (5 ng/ml) and insulin (10 μg/ml). R3 was added at different concentrations 24 hr after seeding. The culture medium containing 0.1% DMSO, with and without R3, was replaced every 48 hr. Values represent means ± SE (n=8).

significant variations of LDH activity were observed even for higher concentrations of R3 (3 μM), which cause complete cell growth arrest (data not shown). This result suggests that R3 exerted no cytotoxic effect on Caco-2 cells.

To determine whether the decrease in cell proliferation was related to a pro-apoptotic process, genomic DNA integrity and phosphatidylserine flipping, 2 major markers of the apoptotic pathway were evaluated. Early apoptotic process is accompanied by an alteration in the organization of phospholipids, leading to the exposure of phospholipids on the cell surface.[24] Detection of phospholipid externalization can be achieved by conjugation with annexin V-FITC. Simultaneously, PI staining was performed. PI enters only in dead cells with a permeable membrane; thus this double staining discriminates viable cells from necrotic and/or apoptotic cells.

After 24 hr of culture in presence of R3 (0.3 μM), the percentage of viable cells (negative to PI staining) decreased from 82% for controls to 77%, and reached 73% in presence of 0.6 μM R3 (Fig. 4). Inversely, the fraction of cells that are positive to PI staining after R3-treatment increased proportionally but harbored a different pattern as compared to dead cells present in untreated controls. Most of the R3-treated cells were in the G2/M transition phase of the cell cycle. Concomitantly, the Annexin V staining profile exhibited no significant differences between R3-treated and untreated samples, suggesting that no early apoptotic cell population appeared in the presence of R3. In addition apoptosis was also assessed by DNA fragmentation measurements, since later stages of apoptosis are usually accompanied by a typical laddering of DNA.[25] Agarose gel electrophoresis of DNA from control and R3-treated cells exhibited that no DNA cleavage occurred when cells were treated with R3 (0.3 to 0.6 μM) for 24 and 48 hr (data not shown). These results indicate that R3 does not induce apoptosis in Caco-2 cells up to 48 hr.

TABLE I – CANCER CELL GROWTH INHIBITION BY R3[1]

Cell lines	Origin	IC_{50} (μM)[2]
Caco2	Human colon adenocarcinoma	0.25
SW480	Human colon adenocarcinoma	0.23
SW620	Metastasic cells derived from SW480	0.20
KB	Human head and neck cancer	0.08
TK6	Human lymphoma	0.10

[1]Inhibition of human cancer cell growth of various origin was assesed following the procedure described in the Material and Methods.–[2]IC_{50}, drug concentration causing a growth inhibition of 50%.

R3 effects on the cell cycle

Exponentially growing untreated and R3-treated Caco-2 cells were subjected to flow cytometry analysis. As shown in Figure 5, treatment with R3 (0.3 μM) caused the accumulation of cells at the G2/M phase of the cell cycle. This accumulation lasted for 16 hr after the first administration of R3. After 48 hr of treatment, the G2/M blockade was not maintained, and the proportion of cells in G2/M was reduced from 54% at 16 hr to 46% at 48 hr. When a second treatment with 0.3 μM R3 was initiated after 48 hr, its effect was exacerbated, leading to a higher proportion of cells blocked at G2/M (77% of the treated cells).

The observed accumulation of cells in the G2/M phase was accompanied by a simultaneous decrease of cells engaged in the G1 phase. The proportion of cells in the S phase was not significantly affected by R3 treatment during the whole experimental period.

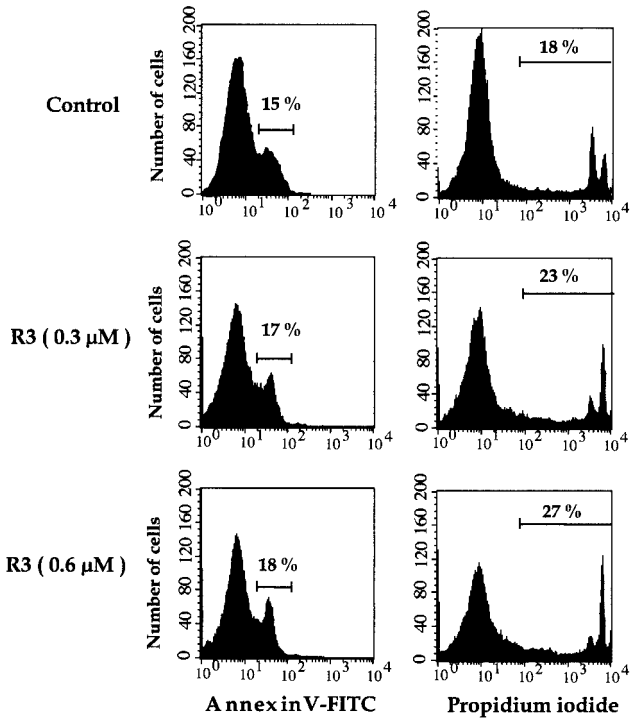

FIGURE 4 – Effects of R3 on phosphatidylserine externalization and cell death. Cells were treated with R3 (0.3 and 0.6 μM) and stained withe Annexin V and propidium iodide. Cells were then harvested and submitted to flow cytometry analysis. The data are representative of 2 separate experiments. Bars represents the percentage of labeled cells.

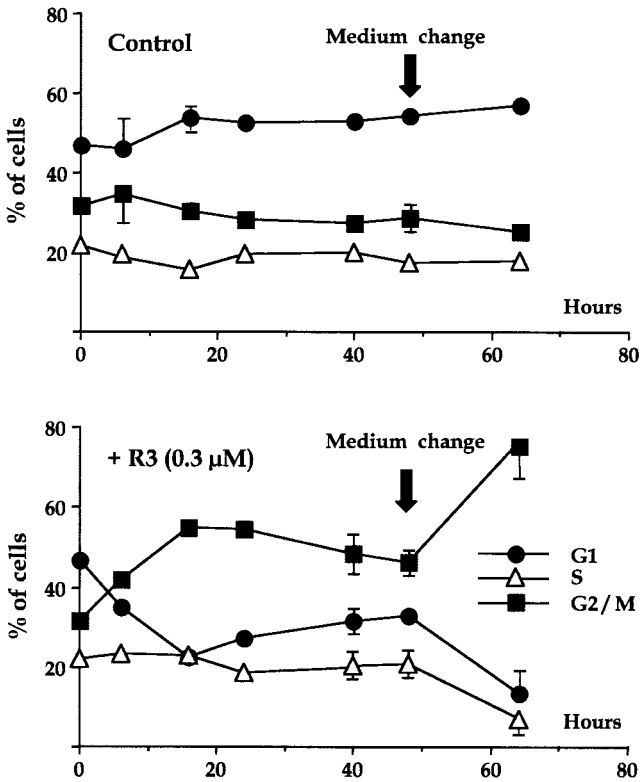

FIGURE 5 – Cell cycle phase distribution of CaCo-2 cells after treatment with R3 (0.3 μM). R3 was added 24 hr after seeding. The culture medium containing 0.1% DMSO, with and without R3, was replaced after 48 hr. Cells were harvested and analyzed by flow cytometry in 3 separate experiments. Values represent the mean ± SE (n=3).

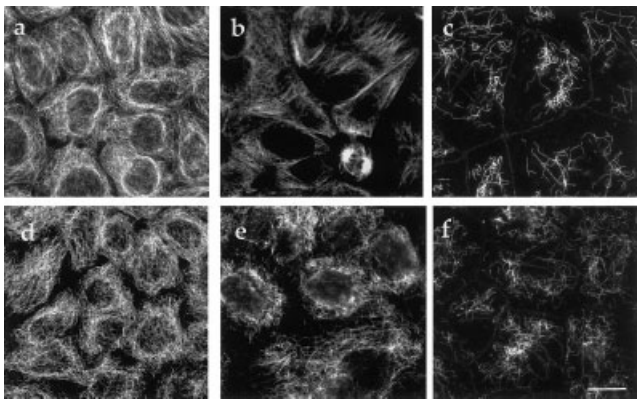

FIGURE 6 – Indirect immunofluorescence staining of microtubules in Caco-2 cells. Cells were cultured for 2 hr in presence of various drugs, fixed and stained with β-tubulin antibodies. (*a*) Untreated cells; (*b*) cells treated with taxol (20 μM); (*c*) cells treated with nocodazole (20 μM); (*d*) cells treated with resveratrol (25 μM); (*e*,*f*) cells treated with R3 (5 and 20 μM, respectively). Bar equals; 20μm.

Effects of R3 on microtubule network and on tubulin polymerization

Immunofluorescent staining of Caco-2 cells, cultured under standard conditions with anti-tubulin antibodies, revealed a dense and well-organized microtubule (MT) network concentrated around the nucleus and extending throughout the cytoplasm (Fig. 6*a*). Taxol induced after 2 hr of exposure hyperpolymerization of MTs and in some cells MTs were organized in bundles (Fig. 6*b*).When cells were treated for 2 hr with nocodazole (20 μM) for 2 hr, the MT network was destroyed (Fig. 6*c*), and short fragments of MTs were randomly distributed throughout the cytoplasm.

Resveratrol (20 μM) treatment of Caco-2 cells for 2 hr had no significant effect on the MT network (Fig. 6*d*). However, treatment of the cells for 2 hr with 5μM R3 caused depolymerization of the MT network (Fig. 6*e*), and most cells contained very short MT spindles. At 20 μM MT disruption was observed (Fig. 6*f*). The effects of R3 on MT organization were similar to those observed with nocodazole (20 μM).

We investigated whether R3 inhibits tubulin polymerization in an *in vitro* assay. Purified tubulin was added in the presence of various concentrations of R3 (up to 30 μM) and turbidity was measured at 37°C for 30 min. The assembly of microtubules was detected by an increased absorbance at 340 nm. As shown in Figure 7, absorbance of control samples increased with time. Addition of R3 prevented tubulin polymerization in a dose-dependent manner and blocked completely MT assembly at a concentration of 30 μM. The inhibitory concentration (IC_{50}) of R3 that reduced the amount of polymerized tubulin by 50% was 4 μM.

Direct interaction of R3 with tubulin was investigated by competition binding to the colchicine binding-domain and to the vinca alkaloïd binding-domain (Fig. 8). R3 had no effect on [^{3}H] vinblastine binding, but inhibited competitively [^{3}H] colchicine binding at concentrations ranging from 0.5 to 5 μM. However, inhibition remained incomplete since 15% of the radiolabeled colchicine remained bound to tubulin, independently of R3 concentrations. Saturation with R3 was obtained at 5 μM.

In order to confirm data obtained with cell extracts, we studied direct interaction of R3 with purified tubulin. As shown in Figure 9, the results obtained with purified tubulin also indicated similar incomplete competition between R3 and colchicine (15% of labeled colchicine remained bound to the purified tubulin). This indicates that the incomplete binding of cochicine to tubulin was not the result of aspecific binding to cellular proteins. These data suggest that R3 either overlaps or partially overlaps with colchicine binding or that R3 binds to a specific site of tubulin.

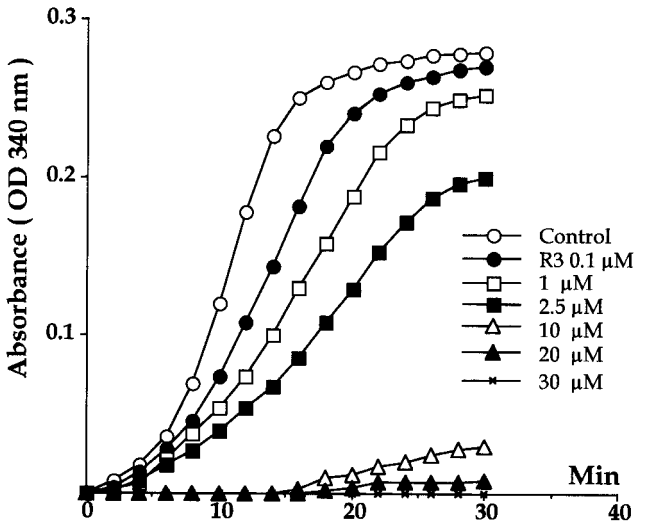

FIGURE 7 – Effects of R3 on microtubule assembly. Purified tubulin proteins in a PIPES buffer containing 1 mM GTP were incubated at 37°C in the absence (control) or presence of various concentration of R3. Polymerisation was initiated by the addition of tubulin. Turbidity was measured at 340 nm.

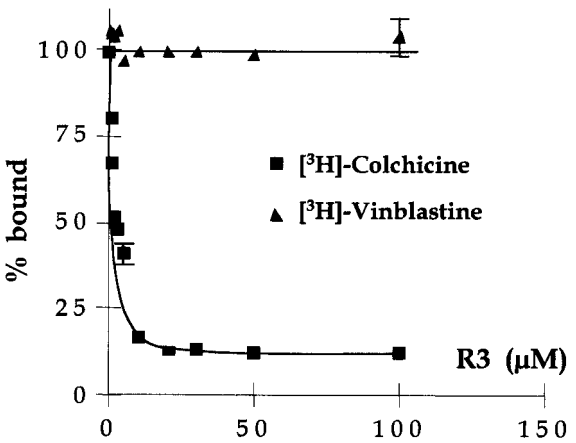

FIGURE 8 – [^{3}H] colchicine and [^{3}H] vinblastine sulphate competition-binding assays with tubulin present in cell extracts. Samples of CaCo-2 high-speed supernatants were spiked with Ring C-[methoxy-^{3}H] colchicine or with [G-^{3}H] vinblastine sulphate and incubated with different concentration of R3 for 60 min at 37°C. Values represent the mean ± SE (n=3).

R3 and polyamine biosynthesis

The polyamines, putrescine, spermidine and spermine are small polycations that are essential for cell growth and differentiation.[26] Polyamines are implicated as regulators of many cellular process including transcription, replication and cell cycle regulation .[27]

Treatment of Caco-2 cells with R3 (0.3 μM) led to a significant decrease of the 2 key enzymes of polyamine biosynthesis, namely, ODC and AdoMetDC (Fig. 10). In cells treated with R3 for 24 hr, ODC activity was reduced by 52%. AdoMetDC exhibited a 20% decrease of activity after 24 hr of treatment, and 40% and 50% after 2 and 3 days, respectively.

As illustrated in Table II, the polyamine content of Caco-2 cells were affected by R3 treatment. These effects were directly related to the lower activity of the 2 rate-limiting enzymes of polyamine synthesis. Putrescine was reduced by 33% after 24 hr, and by 57%

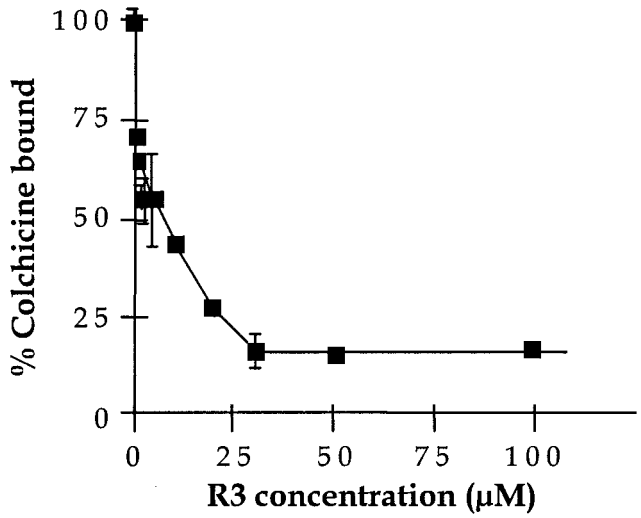

FIGURE 9 – [^{3}H] colchicine competition-binding assay with purified tubulin. Tubulin (5 μM) were spiked with Ring C-[methoxy-^{3}H] colchicine and incubated with different concentration of R3 for 60 min at 37°C. Values represent the mean ± SE (n=3).

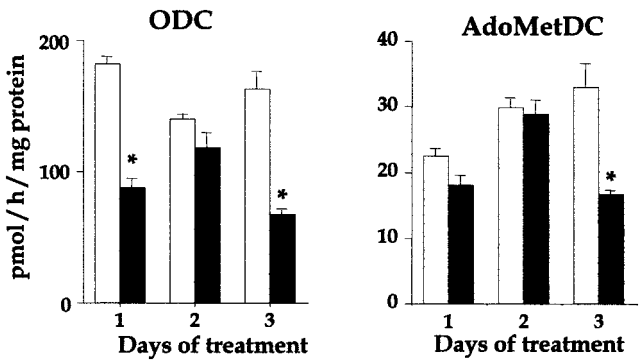

FIGURE 10 – ODC and AdoMetDC activities (pmol/hr per mg of protein) in CaCo-2 cells maintained in culture in the absence (open column) or presence (solid columns) of R3 (0.3μM). The culture medium containing 0.1 % DMSO, with or without R3, was replaced every 48 hr. Results are the mean ± SE of 3 separate experiments; *$p<0.01$.

after 3 days of treatment. Spermidine and spermine concentrations were reduced on day 3 by 60% and 28%, respectively.

DISCUSSION

We investigated the biological properties of the trimethyl derivative of resveratrol (R3). Our data show that R3 inhibited Caco-2 cell proliferation in a dose and time-dependent manner. Cell proliferation was reduced already at a very low concentration of R3 (0.1 μM) and growth was completely blocked at 0.4 μM. The *trans* isomer was 100-fold less effective than the *cis* isomer. This observation is consistent with previous data, indicating that the *cis* isomers of stilbenes are more potent as anticancer agents than their stereoisomeric counterpart.[28] Growth inhibition by R3 was not related to a cytotoxic effect as was shown by LDH release assays. It was associated with the arrest of the cell cycle progression at the G2/M transition phase. The percentage of cells engaged in G2/M increased with a concomitant decrease of the proportion of cells in G0/G1, whereas the number of cells engaged in the S phase remained unchanged. The ability of a drug to block cells in G2/M is consistent with a disruption of the mitotic spindles by interraction with tubulin. In normal cells, the dynamic instability of microtubules is thought to be critically important for normal microtubule functions, including mitosis, cell motility, morphogenesis, axonal transport and secretion.[29,30]

Microtubules have been demonstrated to be a target of numerous cancer chemotherapeutics agents. Unusual stabilization of mitotic spindles by taxol (or paclitaxel), or unusual destabilization by microtubule-disrupting agents such as nocodazole, vincristine and vinblastine,[31] is thought to be a common mechanisms of microtubule poisoning. In the present report, immunofluorescence studies showed that the effects of R3 on the microtubule network are similar to those described for nocodazole, suggesting that R3 is a member of the microtubule-disrupting drugs. Moreover, the *in vitro* tubulin polymerization assays showed that R3 inhibited tubulin polymerization in a dose-dependent manner (IC_{50}= 4 μM); this result was in good agreement with other tubulin-binding agents such as A-204197.[32]

Our data showed also that R3 affected polyamine metabolism by inhibiting the 2 key enzymes involved in polyamine synthesis, leading to a reduction of the intracellular polyamine pool. Polyamines are implicated as regulators of many cellular processes including cell cycle regulation.[27] Therefore the observed polyamine depletion correlates well with the cell cycle blockade observed in the presence of R3. In addition, polyamines may also be involved in microtubule formation, since it has been reported that tubulin polymerization seems to be facilitated by polyamines at physiological concentrations.[33]

Three majors binding sites have been described on the β-subunit of tubulin: the vinca alkaloid binding site, the taxane binding site and the colchicine binding site.[31] We demonstrate here that R3 has no affinity for the vinca alkaloid binding site since R3 did not interfere with the binding of radiolabeled vinblastine on tubulin. In contrast, R3 strongly inhibited the binding of radiolabeled colchicine to tubulin. However, inhibition remained incomplete since 15% of the radiolabeled colchicine remained bound to tubulin, independently of R3 concentrations. Under the same conditions podophyllotoxin a well-known competitive inhibitor of colchicine for binding to tubulin inhibited cochicine binding by 97% (data not shown). Therefore, it seems that R3 overlaps or partially overlaps with cochicine binding to tubulin or that R3 binds to a specific site of tubulin that is not identical with the colchicine binding-site and modifies colchicine binding by allosteric influences. This aspect will be assessed in future experiments, since the evidence of a new binding site on tubulin for R3 would be of great importance for the development of new anti-mitotic drugs.

Most microtubule targeting drugs promote apoptosis of cancer cells. However the process by which anti-microtubule drugs induce apoptosis is poorly understood. Depending on the cell type, the mitotic block induced by anti-mitotic compounds can persist for varying lengths of time, but most cells will exit the cell cycle and undergo apoptosis.[34–36] However we showed that apoptosis of Caco-2 cells was not of major importance in the observed anti-mitotic effect of R3. It is commonly accepted that drugs that influence the microtubule dynamics cause cells to switch into apoptosis when alterations exceed a certain level. It can be hypothesized that in our experimental conditions the doses of R3 were below this critical level. The fact that R3 (at concentrations from 0.3 to 0.6 μM) did not induce apoptosis may also be explained by the p53 status of Caco-2 cells that are harboring a mutated p53 gene.[37] It has been reported that p53 is strongly involved in the apoptotic process triggered by microtubule targeting drugs.[38,39] Inactivation of p53 decreased the sensitivity to paclitaxel and was correlated with a lower induction of apoptosis in human colorectal cells.[40] Another study indicated that by prolonged nocodazole treatment, human glioma cells with p53 mutation, undergo transient arrest at G2/M. These cells subsequently were relieved from mitotic arrest and underwent multiple rounds of DNA replication resulting in hyperploidy.[41] Cells treated with low doses of pac-

TABLE II – EFFECT OF R3 ON THE POLYAMINE CONTENT OF CaCo-2 CELLS[1]

Day of treatment	pmol/mg protein					
	Putrescine		Spermidine		Spermine	
	Control	+R3	Control	+R3	Control	+R3
1 day	447 ± 24	300 ± 31	4,014 ± 75	3,251 ± 133	5,130 ± 151	4,333 ± 342
2 days	337 ± 28	234 ± 16*	4,289 ± 401	2,483 ± 150*	6,015 ± 346	4,783 ± 62*
3 days	465 ± 51	202 ± 19*	4,905 ± 509	1,934 ± 155*	6,953 ± 560	5,070 ± 112*

[1]Cells were exposed to R3 (0.3 μM) 24 hr after seeding, harvested by scraping and extracted with 0.2 N perchloric acid. The culture medium containing 0.1% DMSO, with or without R3, was replaced every 48 hr. The results are means ±SE of 3 experiments. The asterisk indicates a significant difference between control and R3-treated cells, $*P < 0.05$.

litaxel formed multinucleated cells that either became apoptotic in the case of wild-type p53-containing cells or, in the case of p53-deficient cells, exhibited multiple rounds of DNA replication before undergoing apoptosis.[42] Colcemid, another microtubule destabilizing agent, led at concentrations insufficient to cause complete mitotic arrest to the formation of disarranged mitosis with aberrant spindle function.[43] A similar process of "mitotic slippage" may be hypothesized for R3, since incomplete mitotic block was observed after the first exposure of the Caco-2 cells to R3 at a concentration (0.3 μM), which was insufficient to block all the cells in G2/M.

In summary, cancer chemotherapeutic agents that target microtubules have proved to be very useful in the treatment of human cancers. The resveratrol derivative (*Z*-)3,5,4′-trimethoxystilbene (R3) is an interesting anti-mitotic drug that depletes the intracellular pool of polyamines and inhibits tubulin polymerization, which may be useful for the treatment of neoplastic diseases.

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