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Trans- and cis-stilbene polyphenols induced rapid perinuclear mitochondrial clustering and p53-independent apoptosis in cancer cells but not normal cells

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ABSTRACT

We previously reported that 3,4,5,4′-tetramethoxy-*trans*-stilbene (MR-4) induces p53 and perinuclear mitochondrial clustering in cancer cells [Gosslau, A., Chen, M., Ho, C.-T., Chen, K.Y., 2005, A methoxy derivative of resveratrol analogue selectively induced activation of the mitochondrial apoptotic pathway in transformed fibroblasts. Br. J. Cancer 92, 513-521.]. Here we extended the study to over 20 trans-stilbene derivatives and their cis-isomers to explore structure activity relationship. Among them, 3,4,5,4′-tetramethoxy-*cis*-stilbene (MC-4), the cis-isomer of MR-4, was most potent, with IC50 of 20 nM for growth inhibition. MC-4 induced a rapid perinuclear mitochondrial clustering, membrane permeability transition, cytochrome *c* release and DNA fragmentation. To determine whether trans- and cis-stilbene polyphenols may share a common mechanism, we compared the effects of MC-4 and MR-4 in three isogenic cell lines derived from the colorectal carcinoma HCT116 cells: p53+/+ (p53-wt), p53−/− (p53-null) and p21−/− (p21-null). Deletion of either p53 or p21 neither blocked the effects of MC-4 or MR-4 on mitochondrial clustering nor inhibited apoptosis, indicating that the actions of both stilbenes are independent of p53 and p21. Although microtubule disruption has been proposed to account for the action of some cis-stilbene polyphenols, we did not observe differences in microtubule dynamics between cells treated with MC-4 and MR-4. These findings suggest that MC-4 and MR-4 may share a common mechanism whereby the perinucear mitochondrial clustering, rather than p53, p21, or microtubule depolymerization, is critical for their pro-apoptotic action.

1. Introduction

Resveratrol, a trans-stilbene polyphenol isolated from grapes, inhibits cancer cell growth in vitro and tumor progression in mouse models (reviewed in Savouret and Quesne 2002; Aziz et al., 2003; Bhat and Pezzuto, 2002). Recently, resveratrol has also been shown to enhance the life-span in several organisms (Baur and Sinclair, 2006). Combretastatin A-4 is a cis-stilbene polyphenol isolated from *Combretum caffrum*, an African bush willow tree, and is known for its potent antitumor and antiangiogenic activity (Pettit et al., 1987). A prodrug derived from combretastatin A-4 is under clinical trials as an antitumor vascular-targeting agent (Pettit et al., 2005). The wide-range anti-disease activities and potential health benefits of resveratrol and combretastatin have generated a great interest in developing trans- and cis-stilbene polyphenols into useful chemopreventive and chemotherapeutic agents.

Both resveratrol and combretastatin A-4 share a stilbene backbone. However, their respective cellular targets have not been unequivocally established despite their simple chemical structure. Several targets have been proposed for resveratrol and other trans-stilbene polyphenols, including p53/Bax pathway (Lu et al., 2001; Gosslau et al., 2005), CD95-CD95 ligand pathway (Dorrie et al., 2001), or the putative resveratrol-binding proteins (Hsieh et al., 2005). On the other hand, the cellular target of combretastatin A-4 has been thought to be microtubules (Lin et al., 1988; McGown and Fox, 1989). Since the *in vitro* tubulin binding activity of combretastatin A-4 and its analogues is usually two or three orders of magnitude less than their growth inhibitory activity, it has been questioned whether tubulin binding directly account for the cytotoxic effect of combretastatins (Cushman et al., 1992). Indeed, some combretastatin analogues lack tubulin binding activity but still retain potent cytotoxic activity (Borrel et al., 2005).

We have previously reported that 3,4,5,4′-tetrahydroxy-*trans*-stilbene (R-4) and 3,4,5,4′-tetramethoxy-*trans*-stilbene (MR-4) induce p53, Bax and cancer cell apoptosis (Lu et al., 2001; Gosslau et al., 2005). These resveratrol analogues also induce rapid perinuclear mitochondrial

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clustering in transformed WI38VA cells but not in their normal counterparts (Gosslau et al., 2005). We have now extended this study by synthesizing over twenty trans- and cis-stilbene analogues in order to explore structure-activity relationship. Among the stilbene derivatives we tested, 3,4,5,4′-tetramethoxy-*cis*-stilbene (MC-4), the cis-isomer of MR-4, inhibited cancer cell growth with an IC50 of 20 nM. MC-4 and MR-4 were used here respectively as the model compound for cis- and trans-stilbene polyphenols to determine whether they may share common mechanism in inducing apoptosis. We compared the dose–response curve of MC-4 and MR-4 on apoptosis and growth inhibition, and examined the time course of mitochondrial clustering, membrane permeability transition, cytochrome *c* release, and DNA fragmentation. Since mitochondria play a critical role in p53-dependent apoptotic pathway (Polyak et al., 1997) and p53 may be directly involved in the mitochondria-mediated apoptosis (Moll et al., 2005), we investigated possible relationship between p53 activation and mitochondrial clustering by investigating the effects of MC-4 and MR-4 in the p53-null and p21-null colorectal carcinoma cell lines. Our results show that perinuclear mitochondrial clustering represents one of the earliest biological events elicited by both MC-4 and MR-4, and suggest that this event may be responsible for initiating apoptotic cascade in cancer cells. Despite the importance of p53 and p21 in mitochondria-mediated apoptosis, they were not required in the mitochondrial clustering and apoptotic action of MR-4 and MC-4.

2. Materials and methods

2.1. Materials and chemicals

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD). Anti-p53 antibody conjugated to horseradish peroxidase (polyclonal BMG-1B1) was purchased from Roche (Indianapolis, IN), Anti-α tubulin antibody (mouse monoclonal, A-11126) was from Invitrogen (Carlsbad, CA) and anti-cytochrome *c* antibody (mouse monoclonal, 7H8.2C12) from Calbiochem (San Diego, CA). Other biological chemicals were purchased from Sigma (St. Louis, MO).

2.2. Preparation of trans- and cis-stilbenes

The synthesis of resveratrol using 4-methoxybenzyl alcohol and 3,5-dimethoxy-benzaldehyde as the starting materials has been described (Bachelor et al., 1970; Drewes and Fletcher, 1974). Similar strategy was employed to prepare trans- and cis-stilbene polyphenols. Various derivatized benzyl triphenylphosphonium bromides were prepared from corresponding benzyl bromide. Wittig reaction was then performed using various benzaldehyde and appropriate benyl triphenylphosphonium bromide. The identity and purity of each of these compounds has been confirmed by thin layer chromatography, NMR and GC-mass spectroscopy.

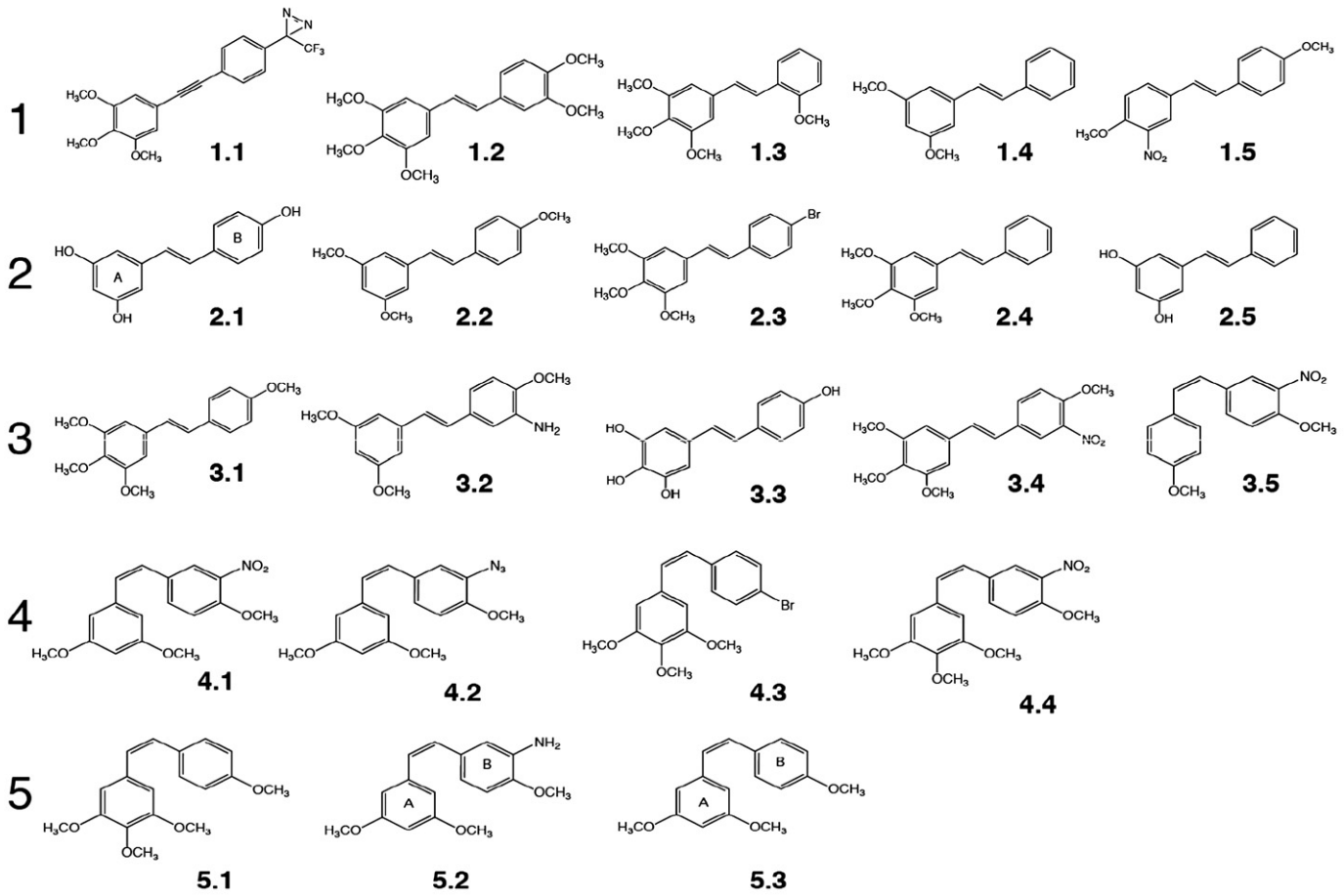

Fig. 1. Structure and activity of cis- and trans-stilbene polyphenol analogues. Resveratrol (2.1) was used as the prototype compound to synthesize trans- and cis-stilbene analogues. The compounds are grouped according to their cytotoxicity. The IC_{50} for compounds in each group is: Group 1, >100 μM; Group 2, 20–80 μM; Group 3, 2–10 μM; Group 4, 0.1–1 μM; Group 5, 20–50 nM. The compounds are referred by a numbering system with the first digit indicating the group that the compound belongs to and the second digit identifying the compound within the group. Compound 1.1, however, is not a bona fide stilbene due to the presence of a triple bond instead of a double bond that connects the two benzene rings.

Table 1
The IC_{50} of stilbene analogues measured with human WI38VA transformed cells

ID#	Compound name	IC_{50} (μM)	Acronym
Group 1			
1.1	3,4,5 trimethoxy 4′-diazirine ethyne	NE	
1.2	3,4,5,3′4′-pentamethoxy trans-stilbene	NE	
1.3	3,4,5,2′-tetramethoxy trans-stilbene	NE	
1.4	3,5-dimethoxy trans-stilbene	NE	
1.5	4,4′dimethoxy, 3-nitro trans-stilbene	NE	
Group 2			
2.1	3,5,4′-trihydroxy trans-stilbene	50	R-3, resveratrol
2.2	3,5,4′-trimethoxy trans-stilbene	25–50	
2.3	3,4,5-trimethoxy 4-bromo trans-stilbene	25–50	
2.4	3,4,5-trimethoxy trans-stilbene	80	
2.5	3,5-dimethoxy trans-stilbene	80	
Group 3			
3.1	3,4,5,4′-tetramethoxy trans-stilbene	2	MR-4
3.2	3,5,4′-trimethoxy 3′-amino trans-stilbene	5	
3.3	3,4,5,4′-tetrahydoxy trans-stilbene	5	R-4
3.4	3,4,5,4′-tetramethoxy 3′-nitro trans-stilbene	10	
3.5	4,4′-dimethoxy 3′-nitro cis-stilbene	10	
Group 4			
4.1	3,5,4′-trimethoxy 3′-nitro cis-stilbene	0.5	
4.2	3,5,4′-trimethoxy 3′-azido cis-stilbene	0.25	
4.3	3,5,4′-trimethoxy 4′-bromo cis-stilbene	0.1	
4.4	3,4,5,4′-tetramethoxy 3′-nitro cis-stilbene	0.1	
Group 5			
5.1	3,4,5,4′-tetramethoxy cis-stilbene	0.02	MC-4
5.2	3,5,4′-trimethoxy 3′-amino cis-stilbene	0.05	
5.3	3,5,4′-trimethoxy cis-stilbene	0.03	

IC_{50} (concentration that gives 50% of growth inhibition relative to the control) was determined by proliferation assay using WI38VA cells. The number represents an average of three measurements with standard errors less than 10%.
NE: no inhibitory effect at 100 μM or higher.

2.3. Cell culture and treatment

All cell lines used in this study were cultured at 37 °C in a humidified, 10% CO_2 atmosphere in DMEM supplemented with 10% FBS. Normal skin fibroblast WI38 and SV40 virally transformed WI38VA cells were used as an isogenetic model for normal and transformed human cells (Lawrence, 2002; Gosslau et al., 2005; Scaglia et al., 2005). HCT116 wild type, p53-null and p21-null cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins School of Medicine). Before experiments, cells were seeded either in culture dishes or in multi-well plates as indicated for the different assays. Stock solutions of all stilbene analogues were made in DMSO. For the control cultures the solvent vehicle DMSO was added to the medium instead of the stock solution.

2.4. Cellular proliferation assays

Cell proliferation was measured by the MTT (3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) method, crystal violet staining, or by cell counting as previously described (Gosslau et al., 2005). Because of the concern that MTT assay may yield false-positive results for certain cell types when treated with flavonoids or polyphenols (Bernhard et al., 2003), we also included the crystal violet dye staining assay in this study (Lu et al., 2001). All three methods yielded comparable results.

2.5. DNA fragmentation assay

The assay was carried out as previously described (Gosslau et al., 2005). Briefly, cells were suspended in buffer containing sodium lauryl sarkosinate (0.5%), proteinase K and RNase A. DNA was extracted with a phenol/chloroform/isopropanol mixture (25:24:1, pH 8.0), analyzed on a 2% agarose gel, stained by ethidium bromide and visualized under UV illumination.

2.6. Caspase activation assay

Caspase activation was analyzed by CaspACE™FITC-VAD-FMK in situ marker (Promega, Madison, WI), a fluoroisothiocyanate (FITC) conjugate of the cell permeable caspase inhibitor VAD-FMK. Cells were seeded on coverslips two days before experiment. At the end of drug treatment, the coverslips were transferred to a 35 mm dish containing 1 ml of medium containing CaspACE-solution. After an incubation of 20 min, cells were analyzed by fluorescent microscopy using an excitation wavelength of 480 nm.

2.7. Rhodamine 123 fluorescence assay

Morphology of mitochondria was monitored using the cationic fluorophore rhodamine 123. Briefly, cells were grown on glass coverslips in 35 mm culture dishes for 2 days. At the end of drug treatment, rhodamine 123 (1.5 mM in medium containing 10% DMSO) was added to the dish to a final concentration of 1.5 μM. After an incubation of 20 min, cells were washed twice with medium and analyzed by fluorescence microscopy with excitation and emission wavelength set at 480 or 525 nm, respectively.

2.8. Mitochondrial potential

Changes in the mitochondrial membrane potential ($\Delta\Psi_m$) was monitored by the Mitochondrial Potential Assay Kit (ATCC, Manassas, VA). Delta-Psi reagent is a lipophilic cation (5, 5′, 6, 6′, tetrachloro-1, 1′,

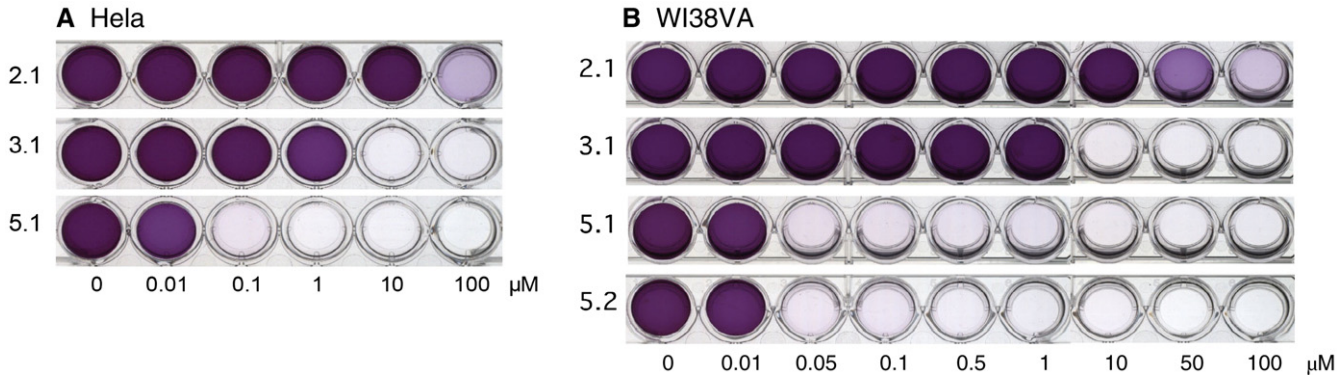

Fig. 2. Antiproliferative effects of trans- and cis-stilbene polyphenol analogues. HeLa cells (A) and WI38VA fibroblasts (B) were subcultured at 1:20 dilution and then treated with stilbene analogues 5.2, 5.1, 3.1 and 2.1 at indicated concentrations. The cell growth was monitored by the MTT assay on day 5 as described in Material and methods section.

3, 3′-tetrathylbenzimidazolyl carbocyanin iodide) that aggregates upon membrane polarization and forms an orange fluorescent compound in intact mitochondria. If the mitochondrial potential is disrupted, the dye cannot access the transmembrane space and remains as green monomers (Cossarizza et al., 1993). Cells were grown on coverslips in 35 mm culture dish for 2 days. At the end of drug treatment, cells on coverslips were incubated with 1 ml of Delta-Psi reagent solution for 20 min, washed and analyzed by phase contrast microscopy and fluorescence microscopy.

2.9. Cytochrome c release and western blot analysis

Cells were treated with MC-4 for various times and then harvested for subcellular fractionation using Dounce homogenizer. The mitochondrial fraction was isolated by a centrifugation at 14,000 ×g for 10 min, and the supernatant was designated as cytosolc fraction. Equal amounts of protein were loaded on SDS-polyacrylamide gel for electrophoresis. The gel was transblotted to a nitrocellulose membrane for western blot analysis using mouse anti-cytochrome *c* antibody and anti-mouse antibody conjugated to horseradish peroxidase as secondary antibody (Amersham Pharmacia). Immunocomplexes were detected with the ECL Plus Western blot detection kit (Amersham Pharmacia).

3. Results

3.1. Trans- and cis-stilbene polyphenol analogues and their antiproliferative activity

Using resveratrol (3,5,4′-trihydroxy *trans*-stilbene) as a prototype we have synthesized over 20 trans- and cis-stilbene analogues (Fig. 1). We have grouped these analogues according to their IC50 values (Table 1). For example, resveratrol (3,5,4′-trihydroxy *trans*-stilbene) in Table 1 is encoded as 2.1, indicating that it belongs to Group 2 which has IC50 in the range of 20–80 μM. Group 5 analogues are the most potent ones with IC50 values comparable to or better than many classical anti-cancer chemotherapeutic agents such as daunorubicin and etoposide. Fig. 2 shows a comparison of antiproliferative activity of a trans- and two cis-stilbene derivatives with that of resveratrol in human cancer cells. The cis-stilbenes, MC-4 (5.1) and 3,5,4′-

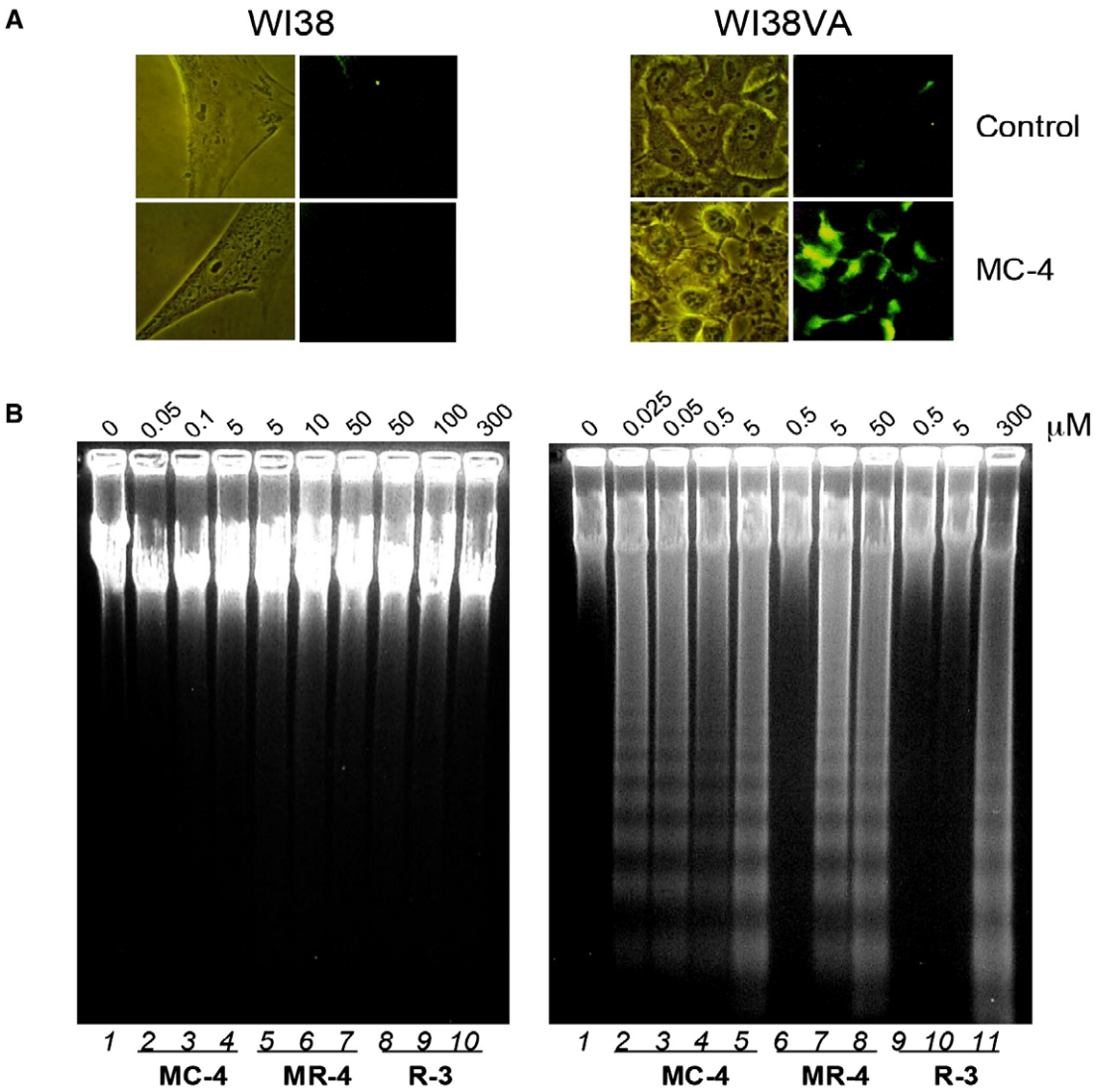

Fig. 3. Differential apoptotic effects of stilbene analogues on normal and transformed fibroblasts. (A) Caspase activation-assay. MC-4 (5.1) was added to the culture of normal WI38 and transformed WI38VA cells for 48 h. Caspase activation was analyzed by incubating the culture with the fluorphore CaspACE™ (FITC-VAD-FMK) for 20 min, cells were examined under a fluorescent microscope. Phase contrast (left panels) and fluorescent micrographs (right panels) were presented for each cell type. (B) DNA laddering analysis. Confluent WI38 or WI38VA cultures were treated with MC-4, MR-4 and resveratrol (R-3) at indicated concentrations for 48 h and processed for DNA isolation and agarose electrophoresis. DNA was visulaized by ethidium bromide staining.

trimethoxy-3′-amino *cis*-stilbene (5.2) are more than 1,000-fold effective than resveratrol (2.1) in inhibiting the growth of cancer cells. MR-4 (3.1), the most potent trans-stilbene derivative in our series, is 200-fold less potent than MC-4 (5.1). In view of their similar structures, it is striking that the antiproliferative activity of these analogues can vary by as much as 3 to 4 orders of magnitude. These results suggest that structure-activity relationship of the stilbene backbone can be further exploited to develop even more potent derivatives. Several features of structure-activity relationship can be derived already: (i) among the six pairs of trans- and cis-isomer, the cis- isomers are always 50–200 times more potent than their trans-counterparts. For example, the IC50 values for isomers 3.1 and 5.1 are, respectively, 5 μM and 20 nM. (ii) An addition of amino group at position C-3′ increases the activity of 3,5,4′ trimethoxy *cis*-stilbene by 2-fold (5.3 vs. 5.2). However, an addition of nitro group at C-3′ reduces the activity of 3,5,4′ trimethoxy cis-stilbene by 10-fold (5.3 vs. 4.1). Similarly, an addition of a nitro group at C-3′ position reduces the activity of 3,4,5,4′ tetramethoxy cis-stilbene by 4-fold (5.1 vs. 4.4). It is likely that an electron-withdrawing group such as nitro-group at C-3′ of a cis-stilbene diminishes its antiproliferative activity. However, an addition of a nitro group at C-3′ position of a trans-stilbene does not affect its activity (e.g. 3.4 vs. 3.1). (iii) An addition of methoxy group at C-3′ position drastically reduces its antiproliferative activity of a trans-stilbene (3.1 vs. 1.2). (iv) The addition of a methoxy group at C-4 enhances the activity for both cis- and trans-stilbenes by at least 4-fold (2.2 vs. 3.1 and 5.3 vs. 5.1) (v) the addition of a methoxy group at C-4′ of a trans-stilbene enhances its antiproliferative activity by at least 10-fold (1.4 vs. 2.2 and 2.4 vs. 3.1). These features, although preliminary, are in line with the notion that the antiproliferative activity of stilbene analogues is due to specific interaction with their cellular targets.

3.2. Apoptosis in transformed human cancer cells

To determine whether trans- and cis-stilbene analogues may share similar mechanism in their anti-proliferative action, we used the stereoisomeric pair, MC-4 and MR-4, as the representative compound for cis- and trans-stilbene polyphenols. We first examined the effect of MC-4 on caspase activation. Fig. 3A shows that the MC-4 induced caspase 3/7 activation in WI38VA cells but not in normal WI38 cells, suggesting that, similar to MR-4, this cis-stilbene specifically induced apoptosis in transformed cells. We then compared the dose–response of MC-4, MR-4 and resveratrol on apoptosis as monitored by DNA fragmentation. Fig. 3B shows that at their respective IC50 concentrations these stilbene analogues induced extensive DNA fragmentation in WI38VA cancer cells, but not in normal WI38 cells. The close correlation between apoptosis and growth inhibition suggests that apoptosis is the key mechanism underlying the anti-proliferative

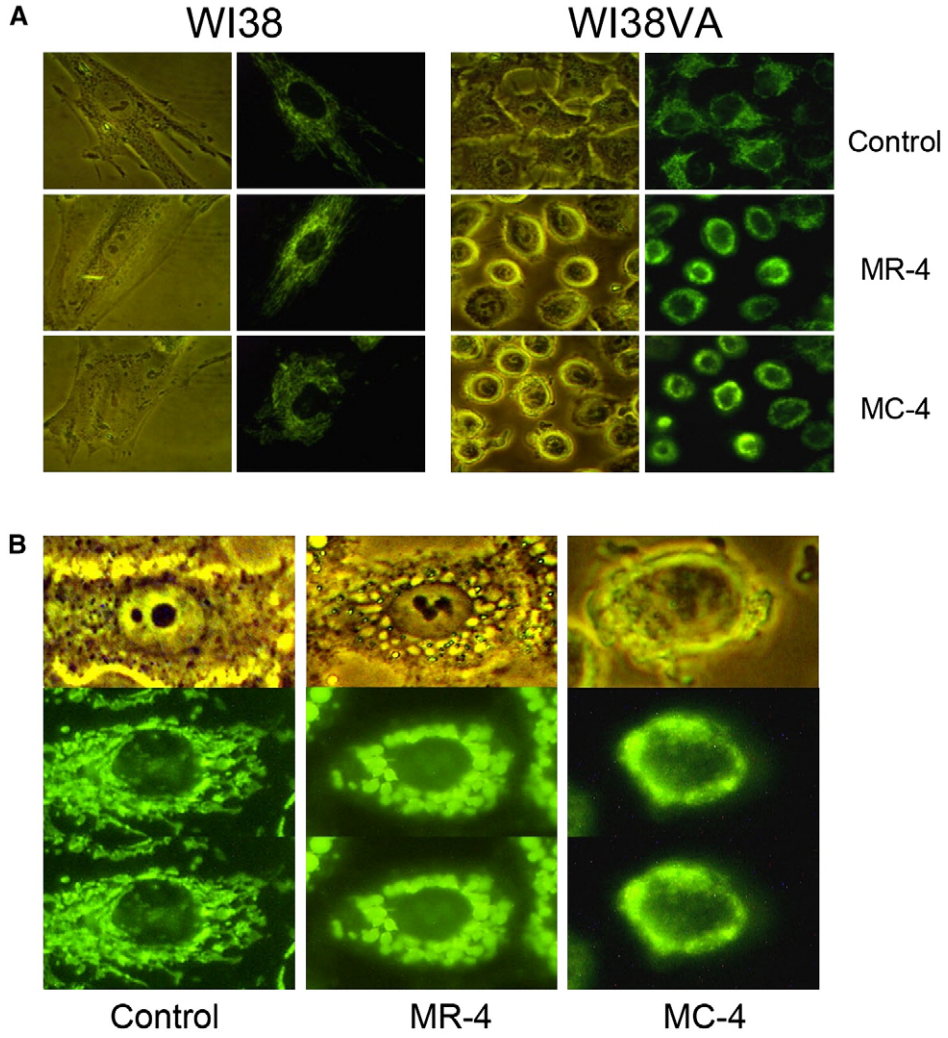

Fig. 4. Mitochondrial distribution in normal and transformed fibroblasts. (A) WI38 and WI38VA cells were treated with MR-4 (50 μM) or MC-4 (50 nM) for 3 h, cells were then stained with rhodamine 123 as described in Materials and methods section and then monitored under a fluorescent microscope. For each cell type, the corresponding phase contrast (left panel) and fluorescence micrographs (right panel) were presented. (B) Pernuclear clustering of mitochondria in WI38VA cells. Cells were treated with MR-4 and MC-4 for 3 h and then examined under phase contrast microscopy (upper panels), and fluorescent microscopy (middle panels). Bottom panels represent merged micrographs.

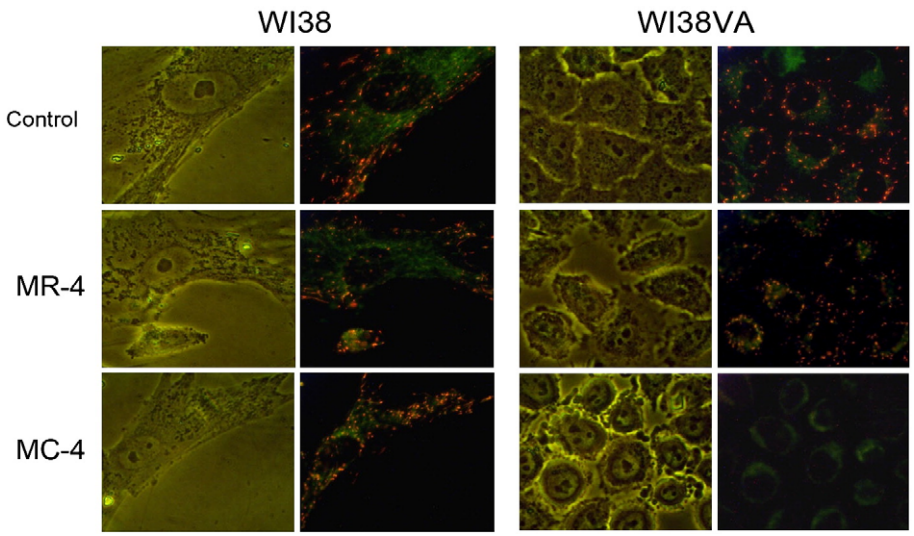

Fig. 5. Effects of MR-4 and MC-4 on Mitochondrial membrane potential in normal and transformed cells. WI38 and WI38VA cells were grown on glass coverslips in a 35 mm culture dish for 2 days. After treatment with MC-4 (50 nM) and MR-4 (50 μM) for 1 h, mitochondrial membrane potential was monitored with Delta-Psi reagent by fluorescence microscopy (excitation wavelength 480 nm) as described in Materials and methods section. For each cell type, the corresponding phase contrast (left panels) and fluorescent micrographs (right panels) were presented.

action of both trans- and cis-stilbene polyphenols. Additional normal and cancer cells, including Caco-2, HeLa, IMR-90SV, IMR-90 and BJ-T were tested and similar results were obtained (data not shown), suggesting that the differential apoptotic effect of stilbene analogues is a general one.

3.3. Effects on mitochondrial morphology and membrane potential

Since MR-4 can cause perinuclear aggregation of mitochondria in cancer cells, we have proposed that mitochondria may be the early target of MR-4 (Gosslau et al., 2005). To determine whether cis-stilbene polyphenols also induce similar mitochondrial redistribution, we compared the effect of MC-4 on the mitochondrial morphology in both normal and cancer cells. Fig. 4A shows that MC-4 elicited a marked perinuclear clustering in WI38VA cells, but had almost no effect in their normal counterparts. Thus, both MC-4 and MR-4 caused perinuclear mitochondrial clustering and apoptosis only in cancer cells but not in normal cells (Fig. 4A vs. Fig. 3B). However, further examination of the mitochondrial morphology in WI38VA cells revealed that the mitochondrial pattern in cells treated with MC-4 was more diffuse than that treated with MR-4. We suspected that depolarization of mitochondria may have already occurred in the MC-4-treated cells. We therefore examined this possibility using a mitochondrial membrane potential probe. Fig. 5 shows that MC-4 caused a collapse of mitochondrial membrane potential, termed membrane permeability transition, within 1 h of treatment. In contrast, MR-4 elicited perinuclear mitochondrial clustering but did not initiate membrane permeability transition. Instead, a longer exposure (>3 h) was required for MR-4 to initiate the onset of membrane permeability transition (data not shown). Neither MC-4 nor MR-4 caused any membrane permeability transition in normal cells, even after prolonged treatment for up to 9 h (data not shown). Thus, although both MC-4 and MR-4 induced rapid perinuclear mitochondrial clustering, the subsequent onset of membrane permeability transition occurred earlier in cells treated with MC-4 than with MR-4.

3.4. Release of mitochondrial cytochrome c

The release of mitochondrial cytochrome *c*, a key event in initiating the mitochondria-mediated apoptosis signaling pathway (Liu et al., 1996), has been shown to be tightly coupled to membrane permeability transition and mitochondrial depolarization (Heiskanen et al., 1999). To determine whether the early action of MC-4 on perinuclear mitochondrial clustering and membrane permeability transition did initiate the apoptotic cascade, we examined the time course of mitochondrial cytochrome *c* release in the MC-4-treated cancer cells. Fig. 6 shows that cytochrome *c* was detectable in the cytosol after 1 h of the treatment with MC-4, consistent with the notion that cytochrome *c* release is coupled to membrane permeability transition. Given that cytochrome *c* release is a hallmark of mitochondria-dependent apoptosis, the early occurrence of mitochondrial redistribution, membrane potential collapse and cytochrome *c* release suggests that mitochondria may serve as the cellular target of MC-4.

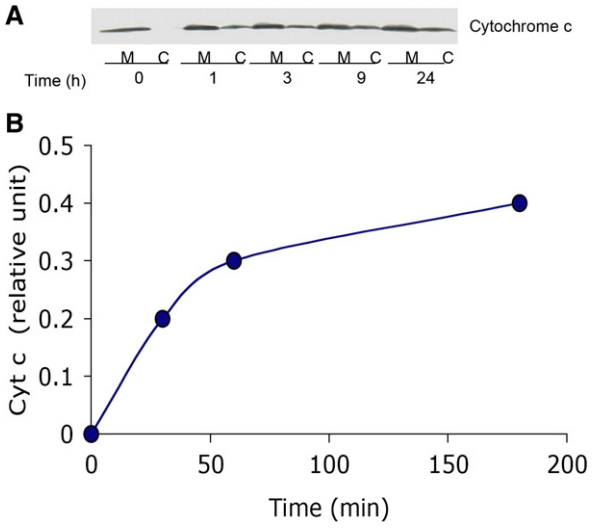

Fig. 6. Effect of MC-4 on the release of mitochondrial cytochrome *c*. WI38VA cells at 80% confluence was treated with MC-4 (50 nM). At indicated time points, cells were harvested for cytosolic and mitochondrial fractionation as described in Materials and methods section. (A) Western blot analysis. Mitochondrial and cytosolic fractions were analyzed by SDS-PAGE. Cytochrome *c* in each fraction was detected by western blotting using mouse anti-cytochrome *c* antibody and peroxidase-conjugated anti-mouse antibody (1:3000). (B) Time course of cytochrome *c* release. Cytochrome *c* in cytosolic fractions was detected by western blotting and quantified. Each data point represented an average of three separate experiments with standard errors less than 10%.

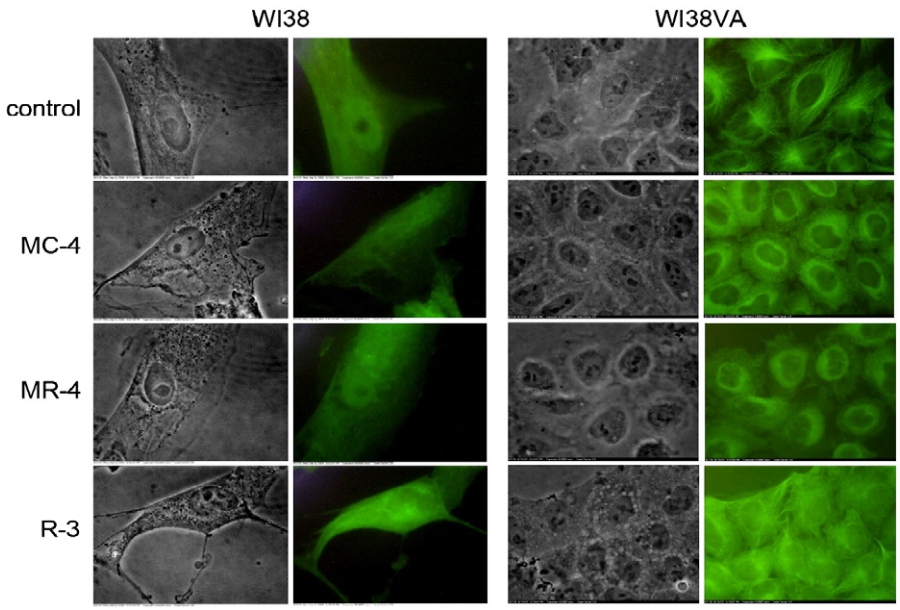

Fig. 7. Effects of MC-4 and MR-4 on microtubule structure in normal and transformed fibroblasts. WI38 and WI38VA cells were treated with MC-4 (50 nM), MR-4 (50 μM), and R-3 (100 μM) for 1.5 h and the microtubule structures were detected with monoclonal anti-alpha tubulin antibody (1:200) and detected with FITC-conjugated goat anti-mouse antibody.

3.5. Effects on microtubules dynamics

It is generally thought that the biological effects cis-stilbene polyphenols, but not their trans-analogues, are due to tubulin binding and microtubule depolymerization (e.g. Gaukroger et al., 2003; Tron et al., 2006). Given that both MC-4 and MR-4 are capable of inducing a rapid perinuclear mitochondrial clustering, we wonder what is the role of microtubules in this process. To address this question, we compared the immunofluorescent staining pattern of microtubules in both normal and cancer cells after the treatment with MC-4 or MR-4. Fig. 7 shows that MC-4, MR-4 or R-3 did not cause appreciable changes of microtubule distribution in normal cells. In cancer cells, treatment with either MC-4 or MR-4 gave similar pattern, which was slightly different from that treated with R-3 or the control. These results indicate that mitochondrial clustering could occur without any significant change in microtubule dynamics, suggesting that microtubule depolymerization may not be directly responsible for causing perinuclear mitochondrial clustering.

3.6. Possible role of p53 pathway in the action of stilbene analogues

Trans-stilbene polyphenols such as resveratrol, R-4 and MR-4 can induce the expression of p53 and p53-driven pro-apoptotic genes (Huang et al., 1999; Lu et al., 2001). Cis-stilbenes such as combretastatin-A4 prodrug has also been shown to induce p53 and p21 (Nabha et al., 2002). In light of the important role of p53 in mitochondria-mediated apoptosis, it is possible that p53 may serve as a common target of both trans- and cis-stilbene polyphenols for initiating the apoptotic cascade. To directly test this possibility, we investigated the biological effects of MC-4 and MR-4 in a set of three isogenetic HCT116 cell lines in which the p53 gene or the p21 gene was disrupted through homologous recombination: p53/p21-wild type, p53−/−, and p21−/− (Bunz et al., 1999). Fig. 8A confirms that the p53-null (p53−/−) cell line does not contain any detectable p53. Fig. 8B shows, to our surprise, that both MC-4 and MR-4 inhibited the growth of the wild type and p53-null cells with similar dose–response and time course. In addition, MC-4 and MR-4 produced similar DNA laddering patterns in both wild type p53 and p53-null cell lines (Fig. 9). Since p21 is a downstream mediator of p53 that can be induced via either p53-dependent or independent pathway ((Halevy et al., 1995; Chin et al., 1995), we also investigated whether p21 is required for the action of MC-4 and MR-4. We found that the absence of p21 did not alter in any way the antiproliferative activity of MC-4 and MR-4 (data not shown). These results suggest that the pro-apoptotic effects MC-4 and MR-4 on cancer cells are independent of p53 and p21.

4. Discussion

Stilbene polyphenols are natural products of biomedical significance and resveratrol and combretastatin A-4 are arguably the founding members of trans- and cis-stilbene polyphenols respectively. The

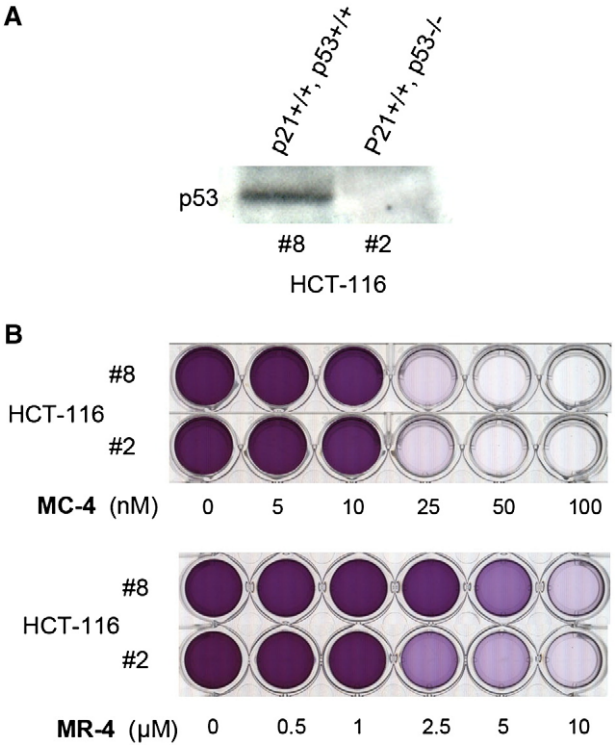

Fig. 8. p53 gene in the action of stilbene analogues. (A) Western blot analysis of p53 in human colon cancer #8 (wild type) and #2 (p53 knockout) cells. (B) Effects of MC-4 and MR-4 on the proliferation of HCT-116 #8 and #2 cell lines as monitored by MTT assay.

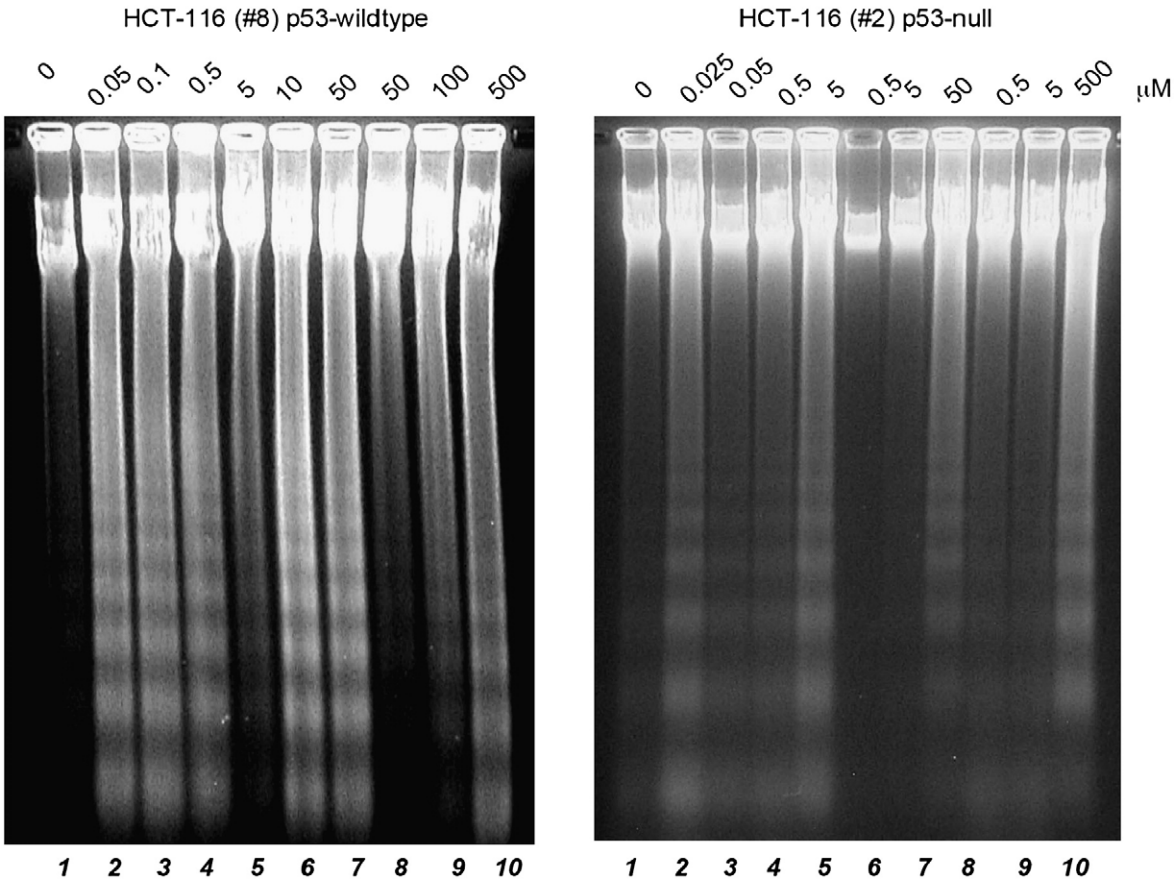

Fig. 9. Apoptotic effect of MC-4 and MR-4 on p53-null HCT116 cells. Confluent HCT-116 p53-wildtype and p53-null cells were treated with MC-4 (lanes 2–4), MR-4 (lanes 5–7) and R-3 (lanes 8–10) at indicated concentrations for 48 h and processed for DNA isolation and agarose electrophoresis. DNA was visualized by ethidium bromide staining.

structure-activity analysis of stilbene analogues (e.g., Pettit et al., 2005; Tron et al., 2006; Hadfield et al., 2005; Fig. 1 and Table 1) has revealed several key features: (i) the cis-stilbene compounds are always more potent than their trans-isomers (Fig. 2); (ii) the 3,4′,5-trihydroxy or 3,4′,5-trimethoxy group appears to be the minimal structural requirement for either trans- or cis-stilbenes to be biologically active; and (iii) the nature and positioning of functional groups on B rings can dramatically affect the biological activity. These features suggest the presence of a specific receptor for stilbene analogues and the high potency of MC-4 may be due to its preferential interaction with this receptor. In this regard it is interesting to note that Wang et al. (2004) have reported the binding of resveratrol to mitochondrial quinone reductase. In addition, in view of the fact that both trans- and cis-stilbene analogues are small molecules containing similar functional groups and that resveratrol uptake is mediated mainly by passive diffusion (Lancon et al., 2004), it is unlikely that the differential effect of stilbene analogues is due to their difference in uptake.

Among the stilbene analogues tested in this study, MR-4 and MC-4 are the most potent trans- and cis-stilbene polyphenol, respectively. (Table 1). MR-4 and MC-4 inhibit cell growth (Fig. 2) and induce DNA fragmentation (Fig. 3). The pro-apoptotic effects of both compounds are limited only to cancer cells, but not to their normal counterparts (Fig. 3). Both stilbenes also cause a rapid perinuclear mitochondrial clustering in cancer cells (Fig. 4), making it one of the earliest detectable events induced by either trans- or cis-stilbene polyphenols. Mitochondrial clustering has been shown to precede cell death in TNF-treated fibrosarcoma cells (De Vos et al., 1998; Desagher and Martinou, 2000). Membrane permeability transition or mitochondrial membrane permeabilization is the decisive event that delimits at cellular level the boundary between life and death (Kroemer et al., 2007). The temporal relationship between loss of mitochondrial membrane potential and cytochrome *c* release has also been established (Heiskanen et al., 1999). In our study, both membrane permeability transition and cytochrome *c* release occur in cancer cells within 60 min after the treatment with MC-4 (Figs. 5 and 6). The findings that MR-4 and MC-4 induce mitochondrial clustering, followed by mitochondrial membrane depolarization and mitochondrial cytochrome *c* release suggest that either mitochondria or cellular sites upstream to mitochondrial clustering may serve as a common target for both trans- and cis- stilbene analogues.

As noted by Tron et al. (2006), not only many combretastatin analogues display discrepancies between cytotoxicity and anti-tubulin activities, but the pharmacodynamic and clinical profile of combretastatins differs from that of other tubulin ibhibitors. Thus, a legitimate question can be raised on whether the cytotoxic effect of combretastatin analogues is dependent on their action on tubulin (Tron et al., 2006). In this regard, our finding that both MC-4 and MR-4 induced rapid perinuclear clustering seems relevant. It is well known that mitochondria move along microtubules as cargoes with the help of molecular motors such as kinesin and dynein and, as such, the cellular distribution of mitochondria is closely linked to microtubule dynamics (Hirokawa, 1998; Yaffe, 1999). Since microtubules have been considered to be the target of cis-stilbene polyphenols, one possible scenario is that MC-4 first induces microtubule depolymerization, which in turn leads to perinuclear mitochondrial clustering. However, this scenario is not applicable to MR-4, since trans-stilbene polyphenols have little or no inhibitory effect on tubulin polymerization. The microtubule distribution monitored by immunostaining does not show significant differences between cell cultures treated with MC-4 or MR-4 (Fig. 7), suggesting that mitochondrial clustering induced by either trans- or cis-stilbene analogues may not be directly related to microtubule

depolymerization. Indeed, it has been reported that microtubule depolymerization actually blocks mitochondrial clustering (De Vos et al., 1998; Kim et al., 2007). However, we do not know at this stage whether kinesin and kinesin-related proteins are involved. Mitochondria are dynamic structures constantly undergoing fusion and fission that are controlled by shaping proteins such as mitofusins 1 and 2, OPA1, drp1 and Fis1 (Okamoto and Shaw, 2005; Chen and Chan, 2005; Chan, 2006). The stilbene analogues may also target at some of these mitochondrial shaping proteins, since disruption of fission can result in perinuclear clustering of mitochondria (Stojanovski et al., 2004). We are currently investigating this possibility. Alternatively, since apoptotic cells tend to shrink, perinuclear clustering of mitochondria could also be a result of simple physical action due to cell shrinkage.

p53 can act as a gatekeeper by controlling cell cycle checkpoints or as a tumor suppressor by modulating the Bax to Bcl-2 ratio and mediating apoptosis (el-Deiry, 1998; Pietenpol and Stewart, 2002). p53 can also directly interact with mitochondria and promote the intrinsic mitochondria-mediated apoptosis (Moll et al., 2005). Abundant literature evidence indicates a close correlation between the up-regulation of p53 and apoptosis in cancer cells treated with trans-stilbene poyphenols (Huang et al., 1999; Gosslau et al., 2005). It is therefore a surprise to find that neither p53 nor p21 is required for the antiproliferative and pro-apoptotic action of not only cis-stilbene MC-4 but also trans-stilbene MR-4 (Figs. 8 and 9). However, it can be noted that resveratrol has been shown to induce apoptosis independent of p53 (Mahyar-Roemer et al., 2001). Taken together, we conclude that p53 or p21 is not the primary target for the pro-apoptotic action of either MC-4 or MR-4. On the other hand, our data suggest that the mitochondrial clustering or the upstream events that lead to mitochondrial clustering could be the common underlying cause for apoptosis induced by either trans- or cis-stilbene polyphenols. Although the mechanism that triggers mitochondrial clustering is still unclear, the time course of its occurrence, as short as 60 min, provides a convenient window for further defining the upstream events and elucidating the mechanism. Finally, the fact that the pro-apoptotic action of both trans- and cis-stilbene polyphenols such as MR-4 and MC-4 is independent of p53 and p21 further emphasizes that stilbene analogues are potentially useful to be developed into chemotherapeutic agents against p53-resistant cancer cells.

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